Corticosterone versus cortisol: distinct roles for endogenous glucocorticoids in human health and disease

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Declaration

I hereby declare that this thesis was written by me, and that, unless expressly stated otherwise, the data published in this thesis are the result of my own work, with the following exceptions:

- Chapter 3
  - Quantification of corticosterone by radioimmunoassay was performed by Ms Lynne Ramage for 1553 of 1923 plasma samples.
- Chapter 7
  - Quantification of non-esterified fatty assays and glycerol in serum was performed by Mr Sanjay Kothiya for 5 of 10 subjects, and insulin for 3 of 10 subjects.
  - Analysis of gene expression in adipose tissue by real-time reverse transcription PCR was performed by Dr Mark Nixon.

The work in this thesis has not previously been submitted for any other degree or qualification.

Scott Mackenzie

Edinburgh, May 2015

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Contents

Abstract

Human plasma contains cortisol (F) and corticosterone (B) at a ratio of ~10:1. B is well studied in mice and rats, which do not produce F due to absent adrenal Cyp17, but is largely neglected in humans. Differential transmembrane export of F>B by ABCB1 may account for accumulation of B in the CNS. Conversely, ABCC1, expressed in human adipose tissue, preferentially exports B>F. Here we tested the hypotheses that: (i) negative feedback suppression of the hypothalamic-pituitary-adrenal (HPA) axis is disproportionately sensitive to B; (ii) adipose tissue is disproportionately sensitive to F; and (iii) low plasma B contributes to impaired HPA axis negative feedback and increased F action in metabolic syndrome.

We validated a stable isotope tracer for B *in vitro* and demonstrated distinct kinetics of B and F *in vivo*. In a randomised crossover study, we undertook ramped steady state infusion of B or F in 10 patients with Addison's disease. Although levels of B were marginally lower than F, ACTH was similarly suppressed, and yet glucocorticoid-responsive transcripts in adipose tissue were much higher following F than B (*PER1* 2.2-fold and *LPL* 1.3-fold; $p<0.05$).

We assessed associations of ACTH-stimulated plasma B and F with features of metabolic syndrome in a cross-sectional study (n=279). Glucose tolerance was impaired with higher F ($\beta=0.146$, $p=0.01$) but lower B ($\beta=-0.056$, $p=0.05$).

These data support the concept of differential tissue sensitivity to B and F, whereby B suppresses the HPA axis more effectively than it induces adverse effects in

adipose tissue. Enhanced CYP17 activity, causing 'relative B deficiency', may contribute to HPA axis activation and enhanced F action in adipose tissue in obesity. B therapy might allow control of HPA axis activation without inducing adverse metabolic effects.

The 'neglected second glucocorticoid', corticosterone, may optimise glucocorticoid action in the human CNS, and simultaneously limit adverse metabolic effects driven by cortisol excess.

Abstracts from this thesis

Oral Presentations

- Low ACTH-stimulated plasma corticosterone may contribute to elevated plasma cortisol in people with metabolic syndrome. Scott D Mackenzie, Rebecca M Reynolds, Jennifer Bolton, James F Wilson, David I W Phillips and Brian R Walker. ENDO 2013, San Francisco, June 16th 2013 (*Endocrine Reviews*, Volume 34, Issue 3 Supplement).
- When two glucocorticoids are better than one: 'relative corticosterone deficiency' in human metabolic syndrome. Scott D Mackenzie, Mark Nixon, Ashley I Taylor, Rebecca M Reynolds, Jennifer L Bolton, Caroline E Hayward, James F Wilson, David I W Philips, Natalie Z M Homer, Ruth Andrew and Brian R Walker. CalSoc 2014, Dunkeld, UK, November 29th 2014.
- Rapid turnover and high free plasma concentrations of corticosterone, the neglected second glucocorticoid, in humans. Scott D Mackenzie, Rebecca M Reynolds, David I W Philips, Anna Anderson, Catriona Kyle, Shaoyun Chen, Geoffrey L Hammond, Lesley A Hill, Natalie ZM Homer, Ruth Andrew and Brian R Walker. ENDO 2015, San Diego, March 6th 2015.

Abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
^{3}H-A	$^3[H]_4$-1,2,6,7-11-dehydrocorticosterone
^{3}H-B	$^3[H]_4$-1,2,6,7-corticosterone
A	11-dehydrocorticosterone
ABC	ATP-binding cassette
ABCB1	ABC transporter B1
ABCC1	ABC transporter C1
ACTH	Adrenocorticotrophic hormone
APCI	Atmospheric pressure chemical ionisation
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
B	Corticosterone
BMI	Body mass index
B/B_0	Ratio of signal at given concentration/signal at concentration = 0
BP	Blood pressure
BSA	Bovine serum albumin
C_0	Concentration at t = 0
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CE	Collision energy
$CHCl_3$	Chloroform
Cl	Clearance
CNS	Central nervous system
CO_2	Carbon dioxide
cpm	Counts per minute
cps	Counts per second

CRH	Corticotropin releasing hormone
CSF	Cerebrospinal fluid
CV	Coefficient of variation
C_{ss}	Steady state concentration
CVD	Cardiovascular disease
CYP	Cytochrome P450
CXP	Collision cell exit potential
D4-cortisol	9,11,12,12-$[^2H]_4$ cortisol
D8-corticosterone	2,2,4,6,6,17A,21,21-$[^2H]_8$-corticosterone
DHEAS	Dehydroepiandrosterone sulphate
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DP	Declustering potential
DOC	11-deoxycorticosterone
E	Cortisone
ECF	Extra-cellular fluid
EDTA	Ethylenediaminetetraacetate
EHERTS	East Hertfordshire cohort study
ELISA	Enzyme-linked immunosorbent assay
EP	Entrance potential
ET2DS	Edinburgh type 2 diabetes study
F	Cortisol
FBC	Full blood count
FCS	Foetal calf serum
G6P	Glucose-6-phosphate
G6P-DH	Glucose-6-phosphate dehydrogenase
GC/MS	Gas chromatography/mass spectrometry
GR	Glucocorticoid receptor
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment – insulin resistance index
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography

HRP	Horseradish peroxidase
HSD	Hydroxysteroid dehydrogenase
IC_{50}	Half maximal inhibitory concentration
IQ	Inter-quartile
IV	Intravenous
K_{el}	Elimination rate constant
K_i	Substrate inhibition constant
K_d	Dissociation constant
K_m	Michaelis constant
K_{cat}	Turnover number
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LDL	Low density lipoprotein
LFT	Liver function tests
LoD	Limit of detection
LoQ	Limit of quantitation
m/z	Mass to charge ratio
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MRM	Multiple reaction monitoring
MW	Molecular weight
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide 2'-phosphate
NADPH	Nicotinamide adenine dinucleotide 2'-phosphate, reduced form
NaOH	Sodium hydroxide
NEFA	Non-esterified fatty acid
O_2	Oxygen
OFN	Oxygen free nitrogen
ORCADES	Orkney complex disease study
P/S	Penicillin/streptomycin
PBS	Phosphate buffered saline
PFP	Pentafluorophenyl
PDL	Poly-D-Lysine

psi	Pounds per square inch
Q	Quadrupole
qRT-PCR	Real-time reverse transcription PCR
R_a	Rate of appearance
RGA	Relative gene abundance
R_i	Rate of infusion
RIA	Radioimmunoassay
RIE	Royal Infirmary, Edinburgh
RMSE	Root mean squared error
RT	Retention time
R_v	Reaction velocity
Scc	Side chain cleavage
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SIM	Selected ion monitoring
SNR	Signal to noise ratio
SPA	Scintillation proximity assay
StAR	Steroidogenic acute regulatory protein
$t_{1/2}$	Half life
TC	Tissue and cell culture
TG	Triglyceride
THA	3α, 5β-tetrahydro-11-dehydrocorticosterone
THB	Tetrahydrocorticosterone
THF	Tetrahydrocortisol
TMB	Tetramethylbenzidine
TFT	Thyroid function tests
UE	Urea and electrolytes
UFC	Urinary free cortisol
UPL	Universal probe library
UPLC®	Ultra performance liquid chromatography
v/v	Volume to volume ratio

V_d	Volume of distribution
WGH	Western General Hospital, Edinburgh
WHR	Waist: hip ratio
WTCRF	Wellcome Trust Clinical Research Facility
w/v	Weight to volume ratio
XIC	Extracted ion chromatogram

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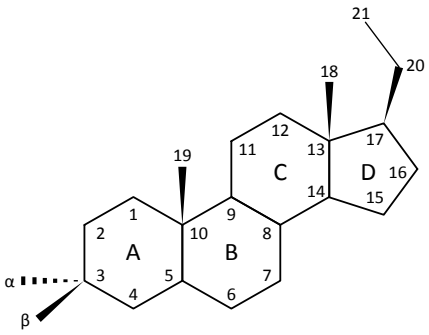
Chapter 1: Introduction

1.1. Physiological Actions of Glucocorticoid Hormones

Glucocorticoid hormones (Figure 1-1) have a wide range of effects on metabolism, CNS function, the cardiovascular system and the immune response (Munck et al, 1984; Sapolsky et al, 2000). The overarching function of glucocorticoid hormones is to co-ordinate the response to actual or perceived threats to homeostasis (stressors). Key elements of this response include maintenance of effective circulating volume (Saruta, 1996; Whitworth et al, 2000); mobilisation of energy stores (Andrews et al, 1999; Macfarlane et al, 2008); inhibition of 'vegetative' functions such as bone formation (Canalis et al, 2002) and reproduction (Whirledge et al, 2010); and regulation of the immune system (Rhen et al, 2005). Reflecting the widespread distribution of the glucocorticoid receptor (GR), up to 20 % of mammalian genes are glucocorticoid responsive (Quax et al, 2013).

Figure 1-1. Basic steroid structure

A carbon skeleton comprising a cyclopentane ring and three cyclohexane rings forms the core of steroid hormones. By convention, steroid rings are identified with capital letters, and carbon atoms are numbered. Substituents and hydrogens are designated according to the number of the carbon atom to which they are attached, and labelled α or β according to their positioning below or above the plane of the page respectively.

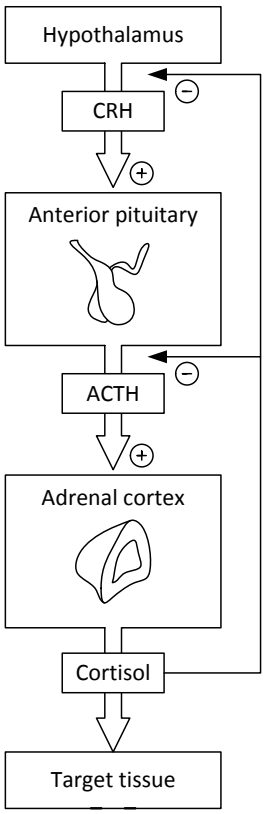


1.1.1. Regulation of glucocorticoid production

Circulating glucocorticoid concentrations show a characteristic rhythmicity and respond rapidly to stressors. This is mediated by neuro-endocrine signalling within the hypothalamic-pituitary-adrenal (HPA) axis. Adrenocorticotrophic hormone (ACTH) is released by the anterior pituitary gland upon stimulation by corticotrophin-releasing hormone (CRH) produced in the hypothalamus. ACTH and CRH secretion (Keller-Wood et al, 1984) is inhibited by glucocorticoids in a classical negative feedback loop (Figure 1-2).

In the adrenal cortex, binding of ACTH to the G-protein coupled melanocortin 2 (MC2R) receptor stimulates cAMP production, which acts upon a cAMP-dependent protein kinase to stimulate steroidogenic acute regulatory protein (StAR). StAR facilitates cholesterol movement into the mitochondria (Stocco et al, 1996) and acutely regulates steroidogenesis by determining supply of substrate. Chronic regulation of steroidogenesis is determined by a number of enzymes whose promoter regions are under regulation by ACTH/cAMP signalling. These include cholesterol side-chain cleavage (P450scc) (Hu et al, 2001), the first and rate-limiting enzyme in steroidogenesis, responsible for quantitative regulation of steroidogenesis. Downstream ACTH/cAMP responsive enzymes modulate steroidogenesis qualitatively, and include 17α-hydroxylase (Rodriguez et al, 1997) and 21-hydroxylase (Kagawa et al, 1992).

Figure 1-2. The hypothalamic-pituitary-adrenal (HPA) axis



Circulating glucocorticoid concentrations peak shortly after waking and fall through the day towards an overnight nadir (Dallman et al, 1993). Superimposed upon this circadian profile is an ultradian rhythm, with pulses of 40-60 minutes (Veldhuis et al, 1989; Young et al, 2001) which effect pulsatile gene expression (Stavreva et al, 2009).

1.1.2. Plasma protein binding

The circulating pool of glucocorticoids is largely bound to the plasma proteins corticosteroid-binding globulin (CBG) and albumin, with only 5-10 % circulating in the free (unbound) state (Hammond et al, 1990). During the stress response, free

cortisol concentrations may be amplified by saturation of CBG; and down regulation of CBG production mediated by neutrophil elastase (Perogamvros et al, 2012).

1.1.3. Intracellular glucocorticoid signalling

While rapid, non-genomic effects of glucocorticoids are mediated by cell-surface receptors (Tasker et al, 2006; de Kloet et al, 2008); the classical actions of glucocorticoids are mediated by effects on gene transcription after activation of intracellular receptors (Chrousos et al, 2005; Nicolaides et al, 2010). Glucocorticoids bind two types of intracellular receptor: the high affinity mineralocorticoid receptor (MR), and the low affinity glucocorticoid receptor (GR) (Reul et al, 1985; Funder, 1997). In the unbound state, GR and MR reside in the cytoplasm bound to chaperone proteins such as heat shock protein 90 (hsp90). Upon binding, the receptor dimerises and translocates to the nucleus (Pratt et al, 2004) where it binds directly to DNA to exert its actions through transactivation of glucocorticoid-response elements (GREs). Alternatively, glucocorticoids may act by an indirect pathway (transrepression), whereby monomeric GR binds to other transcription factors such as NF-κB to inhibit their up-regulation of target genes (Glass et al, 2010).

1.1.4. Mechanisms of tissue-specific glucocorticoid sensitivity

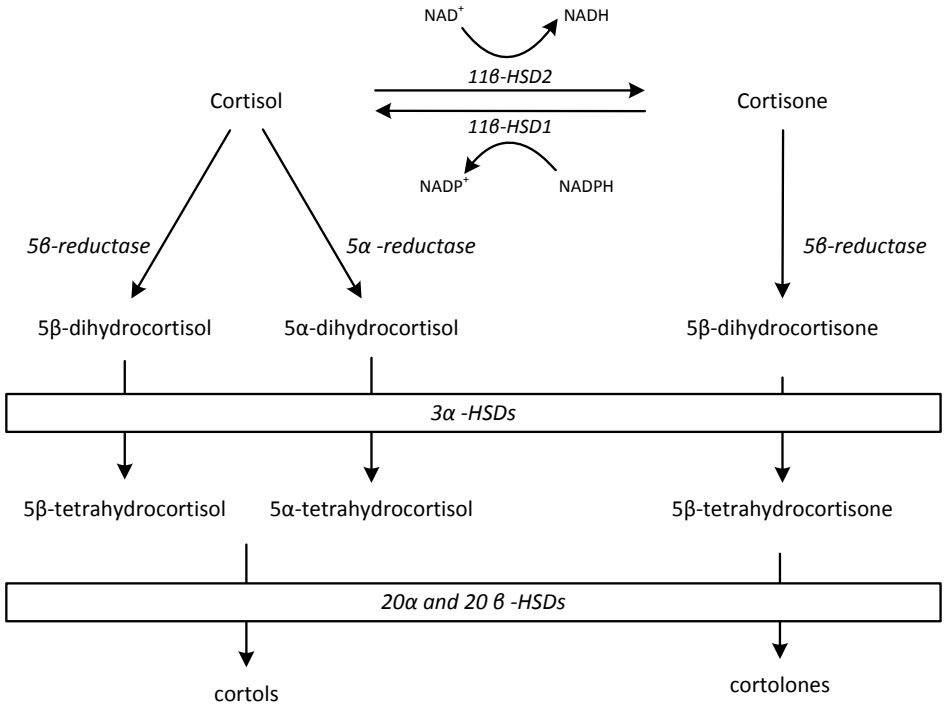
Receptors for glucocorticoids are distributed in a manner that confers varying sensitivity to their action across different tissues. While GR is expressed in almost all tissues, MR is localised to specific tissues including the kidney and hippocampus (Krozowski et al, 1983; de Kloet et al, 1998). MR has a high and roughly equal

affinity (K_d ~ = 0.5-1 nM) for both glucocorticoids and mineralocorticoids (Krozowski et al, 1983; Arriza et al, 1987). However, the circulating concentration of cortisol in humans is approximately three orders of magnitude greater than that of the major mineralocorticoid, aldosterone. To maintain sensitivity to aldosterone in the face of cortisol excess, in tissues such as the kidney and salivary glands, pre-receptor inactivation of glucocorticoids occurs through the action of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Edwards et al, 1988; Funder et al, 1988). 11β-HSD2 catalyzes the nicotinamide adenine dinucleotide (NAD^+) dependent inactivation of cortisol, converting it to cortisone (Figure 1-3). In those tissues where MR is present but significant 11β-HSD2 activity is absent, such as the hippocampus, MR is thought to be readily saturated by glucocorticoid, meaning that regulation of glucocorticoid action by MR may be restricted to conditions of low glucocorticoid production, such as at the diurnal nadir (de Kloet et al, 1998). More recently, it has also been demonstrated that glucocorticoids may act as high affinity ligands for MR outwith the CNS, including in adipose tissue (Caprio et al, 2007), macrophages (Bienvenu et al, 2012) and vascular smooth muscle (Frey et al, 2004). In contrast to MR, the relatively low affinity of GR (K_d ~ = 10-25 nM) for glucocorticoids (Hollenberg et al, 1985) means that expression of GR enables tissues to respond to changes in circulating glucocorticoid concentrations under stress or during diurnal and ultradian peaks (Reul et al, 1985).

Glucocorticoid sensitivity is further modulated through the activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Seckl et al, 2001; Tomlinson et al, 2004). In tissue homogenates, 11β-HSD1 is bidirectional, but in

intact cells the enzyme acts predominantly as a reductase, due to its co-localisation with hexose-6-phosphate dehydrogenase (Atanasov et al, 2008). As such the main action of 11β-HSD1 is to produce cortisol from inactive cortisone, although in some circumstances it may act as a dehydrogenase in intact cells (Bujalska et al, 2002). In contrast to 11β-HSD2, 11β-HSD1 is widely expressed in metabolic tissues including the liver, adipose, skeletal muscle and islets of Langerhans (Chapman et al, 2013). The enzyme acts in an intracrine manner, by regulating access to intracellular receptors; and also in an endocrine manner, by determining relative concentrations of inactive and active glucocorticoids at a systemic level (Chapman et al, 2013).

Figure 1-3. Metabolism of cortisol



1.1.5. Glucocorticoid clearance

In contrast to the reversible inactivation of cortisol by 11β-HSDs, irreversible inactivation occurs primarily in the liver, with direct renal excretion of cortisol accounting for less than 1 % of its clearance (Arlt et al, 2011). The major pathway for hepatic glucocorticoid metabolism begins with the reduction of the A-ring double bond at position 5 (Figure 1-3), catalysed by 5α- and 5β-reductase (Russell et al, 1994; Palermo et al, 2008). Dihydro-metabolites are then further reduced at the keto group by hepatic 3α-hydroxysteroid dehydrogenases (3α-HSDs) to produce tetrahydro-corticosteroids. Water solubility of these metabolites is enhanced by conjugation with glucoronic acid or sulphate, facilitating renal excretion.

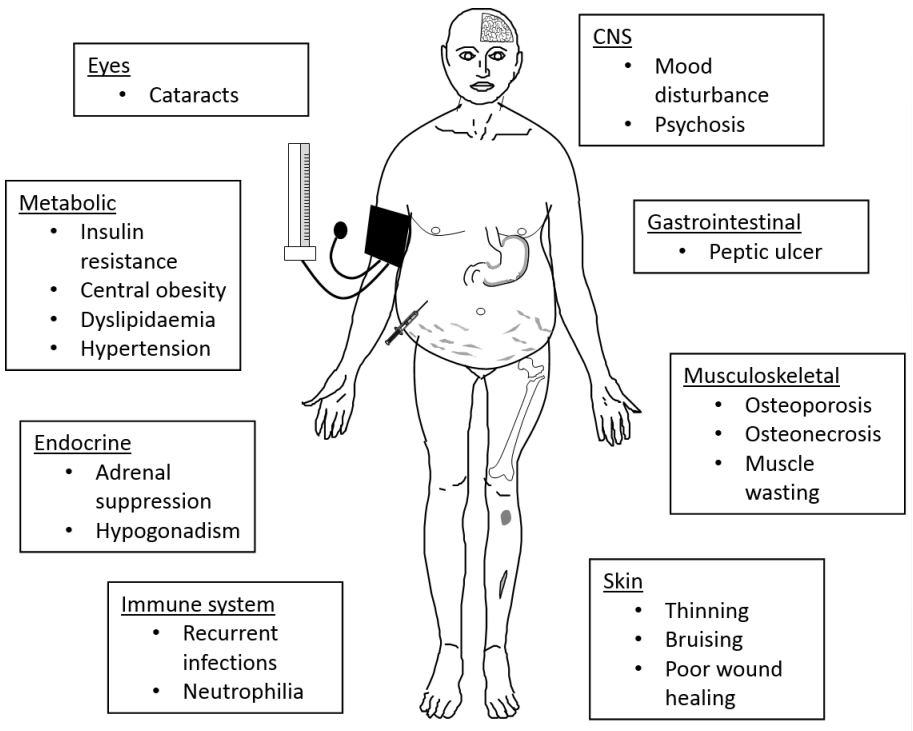
Gas chromatography–tandem mass spectrometry (GC–MS/MS) analysis of 24 hour urinary steroid profiles in healthy individuals (Shackleton, 1986; Arlt et al, 2011; Baudrand et al, 2011) indicate tetrahydro-corticosteroids account for around two thirds of urinary glucocorticoid metabolites, composed of 5α- and 5β-reduced metabolites in broadly equal concentrations. Further reduction of these metabolites by 20-hydroxysteroid dehydrogenases (20 HSDs) yields the cortols and cortolones, which comprise around one quarter of total urinary glucocorticoid metabolites. The remaining metabolites are produced by pathways other than those described above, including hepatic oxidation and hydroxylation reactions.

1.2. Glucocorticoid excess

1.2.1. Cushing's syndrome

In excess, glucocorticoids have a number of adverse effects (Figure 1-4). This is exemplified by Cushing's syndrome, which occurs either as a result of the administration of exogenous glucocorticoids or excess endogenous production. The causes of the latter are an ACTH-producing pituitary adenoma (Cushing's disease), ectopic ACTH production, or autonomous cortisol production from an adrenal adenoma.

Figure 1-4. Clinical features of Cushing's syndrome



1.2.2. Cardiovascular disease and Cushing's syndrome

Among the consequences of overt glucocorticoid excess are a number of metabolic abnormalities, including central obesity, hypertension, dyslipidaemia and insulin resistance (Figure 1-4), each of which is a major risk factors for cardiovascular disease (Hippisley-Cox et al, 2008). Cardiovascular disease is a major cause of premature death in Cushing's syndrome (Pivonello et al, 2005; Ntali et al, 2013; Yaneva et al, 2013). Study of microvascular dysfunction suggests the severity of cardiovascular disease is proportional to the degree of cortisol excess (Fallo et al, 2013). Where chronic glucocorticoid excess is induced through exogenous administration, cardiovascular disease risk is also increased (Souverain et al, 2004; Wei et al, 2004).

1.2.3. Endogenous glucocorticoids and metabolic syndrome

The clinical features of Cushing's syndrome share a number of features with the 'metabolic syndrome', a constellation of risk factors for cardiovascular disease (Reaven, 1988). This observation has led to a body of research which implicates glucocorticoids in the pathophysiology of cardiovascular disease in the absence of overt endogenous glucocorticoid excess or exogenous administration (Walker, 2007).

Definitions for metabolic syndrome have been devised by a number of national and international bodies. The current definition adopted by the Scottish Intercollegiate Guidelines Network (SIGN, 2007) draws upon the recommendations of the American Heart Association/National Heart, Lung, and Blood Institute Scientific Institute (AHA/NHLBI) (Grundy et al, 2005) and the International Diabetes

Federation (IDF) (Alberti et al, 2006) to define metabolic syndrome as any three of the following:

- Increased waist circumference (≥ 102 cm in men and ≥ 88 cm in women; ≥ 90 cm for Asian men and ≥ 80 cm in Asian women), indicating central obesity
- Elevated fasting plasma triglycerides (≥ 1.7 mM)
- Decreased plasma HDL cholesterol (< 1.03 mM for men,< 1.29 mM for women)
- Blood pressure above 130/85 mm Hg or active treatment for hypertension
- Fasting plasma glucose level above 5.6 mM or active treatment for hyperglycaemia.

Although each of these factors is independently associated with cardiovascular risk (Stamler et al, 1986; Stamler et al, 1989; Wilson et al, 2008), insulin resistance is thought to be central to its pathophysiology (Zimmet et al, 1999). Research has focused on the factors which underlie insulin resistance, including the role of glucocorticoid action in metabolic target tissues such as the liver, adipose and muscle.

1.2.4. Associations of cortisol with features of the metabolic syndrome

The majority of studies investigating associations of endogenous glucocorticoids with the metabolic syndrome employ a single (usually morning) measure of plasma cortisol. This has practical advantages compared to urine collections or repeated plasma measurements in large population cohorts. More recently, salivary (Adam et

al, 2009) and hair (Meyer et al, 2012) cortisol have been employed as alternative biomarkers of glucocorticoid exposure.

1.2.4.1. Obesity

In most studies, morning plasma cortisol is weakly inversely correlated with indices of obesity (Phillips et al, 1998a; Walker et al, 2000; Ward et al, 2003; Travison et al, 2007; Reynolds et al, 2010). Study of cortisol pharmacokinetics in humans suggests this is likely to be due to increased clearance of cortisol in obesity (Lottenberg et al, 1998), with evidence from rodent models (Livingstone et al, 2000) and human urinary steroid profiles (Andrew et al, 1998) suggesting this is a consequence of increased activity of 5α-reduction (Russell et al, 1994). Activation of the HPA axis is expected to attenuate any reduction in circulating cortisol concentrations occurring due to increased clearance, with a compensatory rise in cortisol production. Consistent with this, total urinary glucocorticoid metabolite excretion is increased in obesity (Fraser et al, 1999; Reynolds et al, 2001a; Westerbacka et al, 2003). Associations of cortisol with obesity are further complicated by the upregulation of 11β-HSD1 in subcutaneous adipose tissue in obesity (Rask et al, 2001), a finding which has provided a therapeutic target to reduce cortisol action in metabolic tissues (Hughes et al, 2008). However, in the presence of an intact HPA axis this phenomenon is not expected to influence circulating cortisol concentrations.

The weak inverse association of cortisol with obesity carries the potential to obscure associations of elevated plasma cortisol with other cardiovascular risk factors. However, when obesity is adjusted for in multiple regression analyses, elevated

cortisol is associated with a number of features of metabolic syndrome, which shall be considered in turn.

1.2.4.2. Blood pressure

In most studies, morning plasma cortisol correlates positively with blood pressure (Filipovsky et al, 1996; Phillips et al, 1998b; Walker et al, 2000; Ward et al, 2003), although this has not been replicated in all cohorts, perhaps relating to high prevalence of antihypertensive use (Reynolds et al, 2010), or the failure to adjust for body mass index (Maggio et al, 2006). Higher blood pressure is also accompanied by higher morning salivary cortisol (Oltmanns et al, 2006; Abraham et al, 2013; Almadi et al, 2013); while studies of hair cortisol have been inconsistent (Manenschijn et al, 2011; O'Brien et al, 2012).

1.2.4.3. Dyslipidaemia

Although the dyslipidaemia that accompanies metabolic syndrome is well characterised (section 1.2.3), the effects of cortisol on lipid parameters are less clear. In Cushing's syndrome descriptions of the dyslipidaemia resulting from overt glucocorticoid excess are inconsistent (Taskinen et al, 1983; Friedman et al, 1996; Colao et al, 1999), reflecting the complex effects of glucocorticoids on lipid metabolism (Macfarlane et al, 2008).

In most studies, no relationship between morning plasma cortisol and HDL cholesterol has been found (Walker et al, 2000; Maggio et al, 2006; Reynolds et al, 2010). In one study, plasma cortisol was found to positively correlate with HDL cholesterol (Varma et al, 1995), and in others inverse associations between HDL

cholesterol and free cortisol were found in saliva (Almadi et al, 2013) and urine (Fraser et al, 1999). Triglycerides generally show positive associations with plasma cortisol (Phillips et al, 1998a; Walker et al, 2000; Ward et al, 2003), but not in all studies (Maggio et al, 2006; Abraham et al, 2013). Finally, higher total cholesterol has also been found to accompany higher plasma cortisol in individuals with coronary artery disease (Schwertner et al, 1984) and type 2 diabetes (Reynolds et al, 2010).

1.2.4.4. Insulin resistance

A number of studies have demonstrated morning plasma cortisol is positively associated with fasting glucose and the Homeostasis Model Assessment - Insulin Resistance (HOMA-IR) index of insulin resistance (Phillips et al, 1998b; Ward et al, 2003; Reynolds et al, 2010). Similarly, morning salivary cortisol has been found to positively associate with fasting blood glucose (Almadi et al, 2013), and diurnal salivary cortisol studies have revealed cortisol exposure is elevated in type 2 diabetes (Champaneri et al, 2012). Additionally, hair cortisol has been found to be positively associated with glycated haemoglobin in a population-based cohort (Stalder et al, 2013) and in those with type 2 diabetes (Manenschijn et al, 2013).

1.2.5. Associations of cortisol with cardiovascular disease

Evidence from cross-sectional studies suggests these associations between cortisol and metabolic syndrome translate into a higher risk of clinical (Reynolds et al, 2010; Manenschijn et al, 2013) and subclinical (Matthews et al, 2006) atherosclerotic disease. Because cardiovascular disease is less prevalent than its risk factors, it is

likely that many of these studies are not sufficiently powered to characterise associations with 'hard' end-points. However, indices of cortisol exposure are positively associated with coronary atherosclerosis in elective angiography (Troxler et al, 1977) and following myocardial infarction, where they also predict adverse myocardial remodelling (Weir et al, 2011). Additionally, in patients with left ventricular failure, higher plasma cortisol is an independent predictor of increased mortality (Guder et al, 2007). It is possible that these findings reflect reverse causality, whereby cortisol production increases in response to disease severity. Prospective studies are less prone to this bias, and the few such studies which have been undertaken do not confirm an association between plasma cortisol and ischaemic heart disease (Smith et al, 2005; Phillips et al, 2010; Rod et al, 2010). However, these studies have generally failed to include obesity as a confounding variable, other than where it has been included as a co-variable with other features of metabolic syndrome (Smith et al, 2005). Additionally, certainty around the estimates in these studies is low due to the wide variability in plasma cortisol and relatively small number with incident cardiovascular disease.

1.2.6. Evidence of activation of the HPA axis in metabolic syndrome

Accompanying elevated plasma cortisol in metabolic syndrome is evidence of dysregulation of the HPA axis (Pasquali et al, 2006). Tests of HPA axis function employed in clinical practice for the detection of overt glucocorticoid excess, such as the 1 mg oral dexamethasone suppression test, are usually normal in metabolic syndrome (Marin et al, 1992; Stewart et al, 1999; Abraham et al, 2013). Testing of HPA axis suppression using lower doses of dexamethasone has demonstrated subtle

dysregulation of the HPA axis (Ljung et al, 1996; Duclos et al, 2001; Reynolds et al, 2001a), although this finding has not been consistent (Duclos et al, 2005). However, dexamethasone suppression only reflects glucocorticoid receptor mediated negative feedback, and fails to test the contribution to suppression of the HPA axis mediated by cortisol binding to MR in the hypothalamus and hippocampus (de Kloet et al, 1998). The use of a novel test, the Combined Receptor Antagonist Stimulation of the HPA axis (CRASH) test, which employs blockade of feedback mediated by both low affinity (GR) and high affinity (MR) receptors, demonstrates impaired central negative feedback of the HPA axis in obesity (Mattsson et al, 2009). Additionally, dysregulation of central control of the HPA axis is suggested by the finding of impaired habituation of plasma cortisol to the stress of repeated sampling (Reynolds et al, 2001b). Furthermore, responsiveness of cortisol to ACTH and CRH is increased in abdominal obesity (Pasquali et al, 1996); and the cortisol response to wakening is increased (Steptoe et al, 2004). Compensatory activation of the HPA axis in response to increased clearance of cortisol in obesity (section 1.2.4.) might partially explain these findings, but does not explain why elevated plasma cortisol accompanies other features of metabolic syndrome such as hypertension and insulin resistance following adjustment for body mass index.

1.2.7. Origins of HPA axis activation in metabolic syndrome

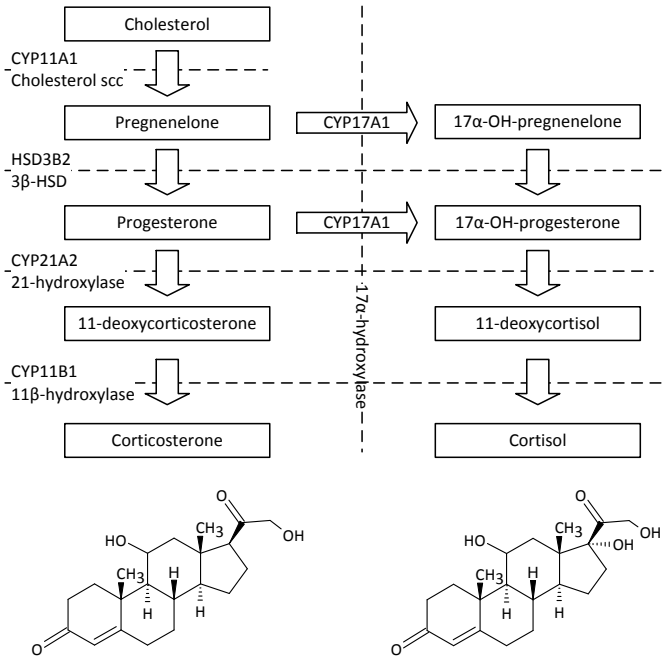
A number of hypotheses have been put forward to explain the factors underlying chronic activation of the HPA axis in metabolic syndrome. Genetic factors explain variability in stress-responsiveness of the HPA axis to a limited extent (Kirschbaum et al, 1992), and initial investigation of environmental factors focused on chronic

psychosocial stress (Bjorntorp, 2001). However, production of cortisol, unlike that of adrenomedullary hormones, is elevated in metabolic syndrome, even after adjustment for psychosocial factors (Brunner et al, 2002). Alternatively, events in early life may lead to 'programming' of the HPA axis resulting in hypercortisolaemia in later life. Low birthweight, reflecting an adverse intrauterine environment, is associated with hypercortisolaemia and metabolic syndrome in adulthood (Phillips et al, 1998a; Levitt et al, 2000; Reynolds et al, 2001a). While the mechanisms behind these associations have not been firmly established, data from animal models suggest that adverse prenatal conditions, accompanied by glucocorticoid excess *in utero*, result in reduced hippocampal GR expression (Levitt et al, 1996), expected to increase HPA axis activation; and that glucocorticoids can mediate changes in GR expression through epigenetic modification (Weaver et al, 2004). The extent to which early life programming, or alternative, as yet uncharacterised, mechanisms account for central 'resistance' to the feedback effects of glucocorticoids in metabolic syndrome remains to be established.

1.3. Corticosterone

To date, study of the role of glucocorticoid action and HPA axis function in metabolic and cardiovascular disease in humans has focused exclusively on cortisol. Little attention has been given to the fact that cortisol is not the only endogenous glucocorticoid in humans. Cortisol production in the human adrenal gland is dependent upon 17-hydroxylation of glucocorticoid precursors in the zona fasciculata (Figure 1-5). However, enzymes expressed in the zona fasciculata which catalyse the final stages of cortisol biosynthesis, 21-hydroxylation and 11β-hydroxylation, are also able to act on 17-deoxy glucocorticoid precursors. As a result, the human adrenal gland secretes both corticosterone and cortisol.

Figure 1-5. Corticosteroid biosynthesis in the human adrenal zona fasciculata



1.3.1. Comparative studies of corticosterone vs cortisol

In the well-studied mouse and rat models adrenal 17-hydroxylase is not expressed (Slaga et al, 1976; van Weerden et al, 1992), and as a result corticosterone is the dominant circulating glucocorticoid. In most large mammalian species, cortisol is the dominant glucocorticoid (Koren et al, 2012), but in some the ratio of corticosterone to cortisol is close to one (Walker et al, 1971; Turner, Jr. et al, 2002), and the dominant glucocorticoid may vary depending upon whether the animal studied is free-ranging or in captivity (Weiss et al, 1966; Weiss et al, 1970; Oddie et al, 1976). Additionally, in animal studies a considerable shift in relative corticosterone and cortisol production has been reported to occur in response to ACTH stimulation (Fevold, 1967; Rosenthal et al, 1993; Vera et al, 2012) and stress (Vera et al, 2011). Therefore, modulation of relative cortisol and corticosterone production occurs in response to environmental factors, perhaps in some part accounting for the wide inter-individual variability in the corticosterone: cortisol ratio between individuals which has been noted regardless of the species studied (Koren et al, 2012).

1.3.2. Adrenal 17α-hydroxylase

The cytochrome P450 enzyme 17α-hydroxylase (CYP17A1) is a key determinant of relative corticosterone and cortisol production. *CYP17A1* is located in chromosome 10q24.3 (Fan et al, 1992) and is expressed in the gonads and adrenal zona fasciculata and reticularis (Missaghian et al 2009). The enzyme acts in the endoplasmic reticulum to transfer two electrons from NADPH via its redox partner, cytochrome

P450 reductase (Akhtar et al, 2005). 17-hydroxylase activity is required for the synthesis of cortisol from pregnenelone and progesterone (Figure 1-5), but the enzyme also catalyses cleavage of the C_{17}-C_{20} bonds of 17α-hydroxypregnenelone and 17α-hydroxyprogesterone, producing dehydroepiandrosterone (DHEA) and androstenedione respectively. In the adrenals, 17-20 lyase activity occurs solely in the zona reticularis, and is responsible for increased production of adrenal androgens from around 8 years of age during adrenarche (Auchus & Miller 1999).

A number of factors are involved in the regulation of CYP17A1 activity. In human adrenocortical cell lines, cAMP-responsive sequences have been identified upstream of the transcriptional initiation site in *CYP17A1*, which bind a number of transcription factors including Sp1, Sp3 and NF-1C (Sewer & Waterman, 2003). The rate of the lyase reaction is augmented in the presence of the endoplasmic reticulum haemprotein cytochrome b_5 (Akhtar et al, 2005). The transcriptional regulation of cytochrome b_5 in the adrenal is similar to that of CYP17A1 (Huang & Miller, 2005). Finally, post-translational modification of CYP17 by cAMP mediated phosphorylation, altered in polycystic ovarian syndrome (Bremner & Miller 2008), is a further determinant of 17, 20-lyase activity (Biason-Lauber et al, 2000).

Mutation in *CYP17A1* (Matteson et al, 1986), results in the rare autosomal recessive condition 17-hydroxylase deficiency (Costa-Santos et al, 2004). Affected individuals usually present at puberty or early adulthood with sex steroid deficiency (Martin et al, 2003), as the maintenance of glucocorticoid production in the form of corticosterone rather than cortisol means they do not experience 'adrenal crisis' in childhood. The association of polymorphism in this gene with circulating

corticosterone and cortisol concentrations in the wider population has not been studied.

1.3.3. Comparison of corticosterone vs. cortisol in humans

1.3.3.1. Basal plasma concentrations

Basal circulating concentrations of corticosterone in humans are around 5-10 % of those of cortisol (Table 1-1). Their reported values vary widely between individuals and according to assay methodology. Early attempts to quantify peripheral venous corticosterone and cortisol concentrations generally employed chromatographic purification followed by measurement with fluorescence, and were poorly specific (Sweat, 1955; Ely et al, 1958). Specificity was improved with more precise chromatographic techniques (Peterson, 1957; Peterson et al, 1960), although the large sample volume required limited their practical value.

The development of radioimmunoassay for corticosterone resulted in improved sensitivity, reduced sample volume requirements and the ability to undertake repeated measurements on a larger scale. However, a limited number of studies have reported plasma corticosterone concentrations in humans, and the number of subjects in each study is small. In most studies, morning plasma corticosterone concentrations are in the region of 10-20 nM, a notable exception being the study undertaken by Raubenheimer and colleagues (Raubenheimer et al, 2006), in which elevated corticosterone (58.4 ± 9.2 nM) and cortisol (830.4 ± 68.4 nM) were found in individuals undergoing lumbar puncture, suggesting a stress response.

Interpreting the results of these studies requires knowledge of the cross-reactivity of the antibody used in the assay. This is of particular importance for cross-reactants present at high concentrations, such as cortisol, for which cross-reactivity of 2.8 - 7.8 % has been reported (West et al, 1973; Nabors, Jr. et al, 1974; Nishida et al, 1976). In such cases, chromatographic separation of analyte and cross-reactant has been employed to minimise assay interference due to cross-reactivity.

Associations between gender and basal corticosterone concentrations have been reported but these have not been consistent (Huther et al, 1970; Nabors, Jr. et al, 1974; Schoneshofer et al, 1977; Wilens et al, 1984). In females, concentrations may be increased in the luteal phase of the menstrual cycle (Schwartz et al, 1975; Schoneshofer et al, 1977), although this may be related to cross reactivity with progesterone, which is as high as 44% (Nabors, Jr. et al, 1974).

Liquid-chromatography mass spectrometry (LC-MS) is a highly sensitive and specific technique which has become the gold standard for quantitative steroid analysis (Shackleton, 2010). Measurement of plasma corticosterone by LC-MS in a small cohort (Karssen et al, 2001) resulted in measured concentrations that were similar to those reported in studies using radioimmunoassay.

Table 1-1. Summary of studies describing of plasma corticosterone (± cortisol) concentrations in healthy subjects

Reference	Gender (M/F)	Age (years)	Corticosterone (B) nM	Cortisol (F) nM	B/F‡	n	Time	B assay Technique
Sweat, 1955	ns	ns	124.1 ± 14.4	298.0 ± 16.6	0.416	21	ns	Fluorometric method
Peterson, 1957	18/12	ns	31.7 ± 11.5	386.2 ± 33.1	0.082	30	ns	Isotope dilution/fluorescence
Ely et al, 1958	ns	ns	86.6 ± 5.8	300.7 ± 15.1	0.288	20	ns	Fluorometric method
Peterson et al, 1960	ns	ns	28.9 (23.1-51.9)			20	ns	Isotope dilution/fluorescence
Fraser et al, 1968	ns	ns	19.0 (3.8-66.4)	270.3 (85.5-557)	0.067	29	ns	Double isotope assay
Huther et al, 1970	M	26.8 ± 2.6	47.2 ± 6.0	403.0 ± 37.5	0.124	9†	1045-1145*	Fluorometric method
	F	31.3 ± 4.4	49.7 ± 6.5	422.4 ± 67.8	0.118	10†		
Dluhy et al, 1972	8/2	21-34	26.3 ± 3.2	634.5 ± 55.2	0.041	10	0900	Radioimmunoassay
Newsome, Jr. et al 1972	ns	ns	11.5 ± 0.9	339.3 ± 22.1	0.034	8	ns	Competitive protein binding
Oddie et al, 1972	ns	ns	12.1 ± 2.6	383.4 ± 4.0	0.030	18	0900	Double isotope assay
West et al, 1973	M	19-50	11.4 ± 1.7	386 ± 36	0.030	15	0800	Radioimmunoassay
	F[1]		20.4 ± 2.0	386 ± 36	0.053	9		
	F[2]		16.7 ± 2.1	359 ± 21	0.047	9		
Meikle et al, 1974	ns	20-55	13.7 ± 2.6			11	0800	Radioimmunoassay
Schwartz et al, 1975	F[1]	ns	10.4 ± 3.7			5†	0800-1000	Radioimmunoassay
	F[2]		15.9 ± 6.8			5†		
Nishida et al, 1977	ns	ns	23.0 ± 3.0	419.6 ± 28.7	0.055	10	0900	Radioimmunoassay
Schoneshofer et al 1977	M	ns	12.2 ± 1.1			31	0800-1100	Radioimmunoassay
	F[1]		7.0 ± 1.1			16		
	F[2]		12.7 ± 1.7			15		
Kobayashi et al, 1979	ns	ns	6.9 ± 2.6			20	0930	Enzyme immunoassay
Wilens et al, 1984	ns	ns	11.7 ± 0.9			10	0800	Radioimmunoassay
			4.4 ± 0.6			10	1600	
Karssen et al, 2001	M	57 ± 1.9	16.4 ± 3.1	350.0 ± 81.5	0.047	11	ns	LC-MS
Raubenheimer et al, 2006	M	39.2 (23-70)	58.4 ± 9.2	830.4 ± 68.4	0.069	16	0830-0900	Radioimmunoassay

Mean ± SEM or range (). ns = not specified

† = mean of 5 samples for each subject * = approximate time inferred from clinical protocol

[1] follicular phase, [2] luteal phase, ‡ mean [B] (nM)/mean [F] (nM)

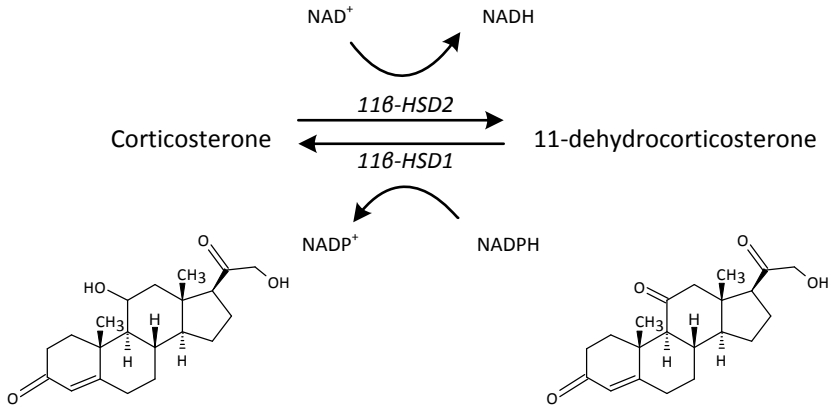
1.3.3.2. Regulation of corticosterone production by the HPA axis

Like cortisol, circulating corticosterone concentrations peak upon waking and fall through the day (Peterson, 1957). Ultradian pulses are also synchronised with those of cortisol (West et al, 1973; Nabors, Jr. et al, 1974; Vagnucci et al, 1974). Despite this synchronicity, cortisol and corticosterone production respond differentially to HPA axis signalling. Under ACTH stimulation, corticosterone shows a consistently exaggerated response, with a 2-4 times greater increment (as determined by the ratio of stimulated: basal concentrations) in comparison to that of cortisol (Peterson, 1957; Peterson et al, 1960; Fraser et al, 1968; Nabors, Jr. et al, 1974; Nishida et al, 1977; Ganguly et al, 1977). Similar findings have been reported following surgical stress (Hamanaka et al, 1970), and insulin-induced hypoglycaemia (Fraser et al, 1968). Finally, suppression of the HPA axis using the synthetic glucocorticoid agonist dexamethasone also results in a rise in the ratio of plasma corticosterone to cortisol (Newsome, Jr. et al, 1972; Nishida et al, 1977), perhaps explained by inhibition of CYP17 by dexamethasone (Lee et al, 1999).

1.3.3.3. Pre-receptor metabolism

Cortisol and corticosterone are both metabolised by the 11β-hydroxysteroid dehydrogenases (11β-HSDs), which catalyse their interconversion between active and inactive forms (section 1.1.4). Similar to the NAD^+-dependent conversion of cortisol to cortisone, 11β-HSD2 catalyses the inactivation of corticosterone by formation of a keto group at position 11, to produce 11-dehydrocorticosterone (Figure 1-6).

Figure 1-6. Metabolism of corticosterone by the 11β-hydroxysteroid dehydrogenases (11β-HSDs)



Determination of the Michaelis constant (K_m) of human 11β-HSD2 points to the enzyme having a greater affinity for corticosterone over cortisol (Figure 1-7 i). However, Michaelis-Menten plots generally reveal that at glucocorticoid concentrations equivalent to basal concentrations of corticosterone, inactivation of both glucocorticoids by 11β-HSD2 proceeds at approximately similar rates. Under ACTH stimulation (section 1.3.3.2), corticosterone might be expected to reach a level sufficient to saturate 11β-HSD2, although in most circumstances it appears likely that, similar to its effects on cortisol, the enzyme would prevent significant corticosterone-mediated MR activation in the distal nephron.

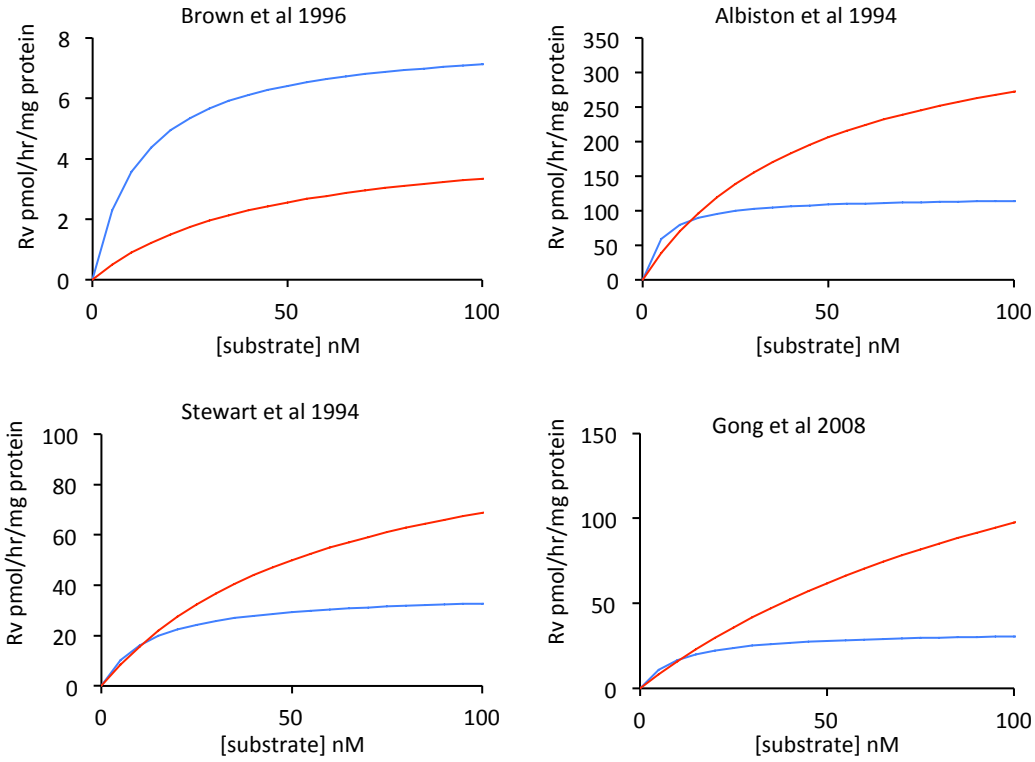
Activity and directionality of 11β-HSD1 depends on whether intact cells or homogenates are studied. Direct comparison of the reductase activity of the enzyme for 11-dehydrocorticosterone and cortisone has been studied in both (Maser et al, 2002; Arampatzis et al, 2005). Unlike 11β-HSD2, the K_m of 11β-HSD1 for both 11-keto steroids is within the supraphysiological range, and across the range of concentrations studied, production of cortisol exceeds the rate of production of

corticosterone for a given concentration of inactive 11-keto substrate (Figure 1-7 ii). As such, in tissues where the enzyme is expressed, 11β-HSD1 is likely to amplify corticosterone-mediated glucocorticoid signalling to a lesser extent than that of cortisol. Dehydrogenase activity has been studied in liver (Maser et al, 2002), where it has been found to be greater for cortisol than for corticosterone, and in the kidney (Gong et al, 2008), where little difference was found between the two substrates.

Figure 1-7. Metabolism of corticosterone and cortisol by human 11β-hydroxysteroid dehydrogenases (11β-HSDs)

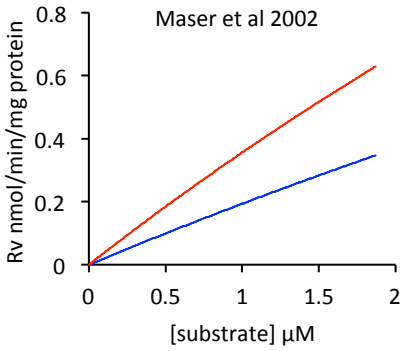
Summary of studies comparing kinetic properties of 11β-HSDs for corticosterone (B) and cortisol (F). 11β-HSD2 (figure i) acts as a dehydrogenase, catalysing the conversion of the active glucocorticoids B and F to inactive 11-dehydrocorticosterone (A) and cortisone (E) respectively. 11β-HSD1 (figure ii) acts predominantly as a reductase, converting B and F to A and E respectively; but also as a dehydrogenase, catalysing the reverse reactions. Modelled enzyme kinetics are derived from data in tables according to the Michaelis-Menten equation: $v = (Vmax\times\ [S])/(Km + [S])$. Red line refers to interconversion of F and E; blue line refers to interconversion of B and A. Rv = reaction velocity. Data refer to mean (± SEM where available).

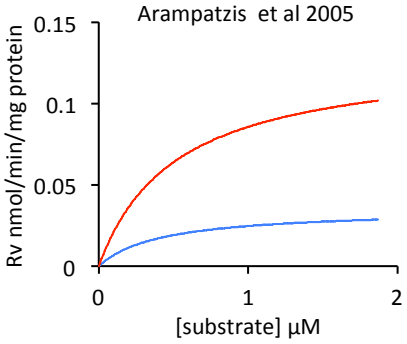
i) 11β-HSD2	Reference	Brown et al, 1993	Brown et al, 1996	Albiston et al, 1994	Stewart et al, 1994	Gong et al, 2008
Substrate	Source of enzyme	partially purified human placenta	transfected CHO cells	transfected CHOP cells	foetal kidney microsomes	renal cortex homogenate
B	K_m (nM)	14 ± 1	12.4 ± 1.5	5.1 ± 0.7	13	10.2
	V_{max} (pmol/hr/mg protein)		8.0 ± 0.7	120	37	33.6
F	K_m (nM)	54 ± 14	43.9 ± 8	47 ± 1.0	60 ± 5	134.2
	V_{max} (pmol/hr/mg protein)		4.8 ± 0.7	400	110 ± 4	228



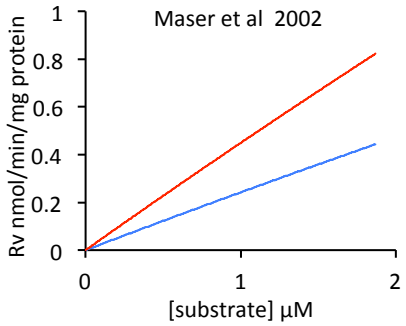
ii) 11β-HSD1	Reference	Maser et al, 2002		Arampatzis et al, 2005		Gong et al, 2008	
	Source of enzyme	Purified liver protein		Transfected HEK-293 cells		Renal cortex homogenate	
Reaction	Substrate	K_m µM	V_{max} nmol/min/ mg protein	K_m µM	V_{max} nmol/min/mg protein	K_m µM	V_{max} nmol/min/ mg protein
Reductase	A	19.7	4.0	0.420 ± 0.050	0.035 ± 0.004		
	E	13.9	5.3	0.519 ± 0.041	0.130 ± 0.013		
Dehydrogenase	B	42.8	10.6			0.42	0.0021
	F	41.3	19.0			3.31	0.0062

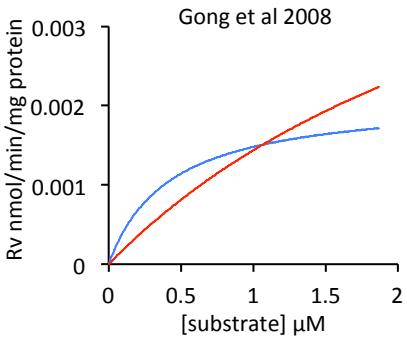
Reductase





Dehydrogenase





1.3.3.4. Production and clearance

Circulating concentrations of cortisol and corticosterone are determined by their rates of production and clearance (Table 1-2). Corticosterone production rate has been estimated using the principle of isotope dilution, with infusion of [^{3}H]-labelled corticosterone, administered as a bolus dose (Peterson et al, 1960), or steady state infusion (Huther et al, 1970). Although these studies were limited by non-specific assay methodology, the estimated corticosterone production rate was similar in each study, at around 0.6 µmol/hr, or 14 µmol per 24 hours, and cortisol production rates

determined simultaneously were similar to those of later studies using radio-isotope tracers (Zumoff et al, 1974), albeit slightly higher than more recent estimates derived from LC-MS analysis of stable isotope tracers (Esteban et al, 1990).

In comparison to the 10-20 fold difference in circulating concentrations, the 3-4 fold difference in production rates points to differential clearance of corticosterone and cortisol. Indeed enhanced clearance of corticosterone relative to cortisol has been reported in studies in which radiolabelled isotopes of both glucocorticoids have been co-infused at steady state (Huther et al, 1970; Messerli et al, 1976). More rapid clearance of corticosterone is likely to reflect differential affinities of hepatic A-ring reductases for the two substrates (Chen et al, 2011) and less efficient regeneration of corticosterone by 11β-HSD1 (section 1.3.3.3). More rapid clearance of corticosterone accounts at least in part for its shorter half-life in comparison to cortisol (Ely et al, 1958; Raubenheimer et al, 2006). However, it is likely that this is also determined by a lower apparent volume of distribution (V_d): following administration of equivalent bolus doses (Raubenheimer et al, 2006), the peak plasma concentration of corticosterone is approximately 3-fold greater than that of cortisol.

Estimates of half life and volume of distribution in the studies above are derived by modelling plasma glucocorticoid concentrations according to a single compartment model. However, when varying doses of corticosterone were infused, pharmacokinetic properties were found to vary widely according to the dose administered (Peterson et al, 1960), suggesting more complex pharmacokinetic modelling is required. Although a single compartment model was employed by

these authors, the data reveal an early rapid decline, which precedes the monoexponential phase studied by the authors, suggesting the presence of a second compartment (Peterson et al, 1960). In studies of cortisol pharmacokinetics following intravenous bolus administration, there is considerable inter-individual variability, and while some have found a single-compartment model best describes their data (Bright, 1995; Perogamvros et al, 2011), others suggest a two compartment model is more appropriate (Toothaker et al, 1982; Thomson et al, 2007). The presence of a second compartment implies that release of glucocorticoids from binding proteins, and subsequent distribution into target tissues, is not instantaneous. Corticosterone binds to CBG with an affinity that is high and roughly equal to that of cortisol (Stroupe et al, 1978; Dunn et al, 1981). Where higher doses are infused, and proportionately less glucocorticoid expected to be protein-bound, delayed equilibration of free hormone from the peripheral compartment to the central compartment could explain why 'pharmacological' doses of corticosterone (Peterson et al, 1960) and cortisol (Toothaker et al, 1982) have a longer half life and higher apparent volume of distribution than 'physiological' doses.

Table 1-2. Production and clearance of corticosterone and cortisol reported in previous studies

	Corticosterone	Cortisol	n	Gender (M/F)	Age (years)	Design	Isotope	Dose	Corticosterone assay Technique	Reference
Production rate (µmol/hr)	0.52 ± 0.06		7	4/3	30 ± 7	Bolus	^{3}H	7-30 µg	Isotope dilution/fluorescence	Peterson et al, 1960
	0.55 ± 0.11	1.93 ± 0.26	18	8/10	31 ± 3	Steady state	^{3}H	<0.037 µg/hr	Fluorometric method	Huther et al, 1970
Half life (mins)	76.2 ± 18		17	ns	ns		Unlabelled	100 mg	Isotope dilution/fluorescence	Peterson et al, 1960
	30	97	8	ns	ns	Bolus	Unlabelled	2 mg/kg	Fluorometric method	Ely et al, 1958
	55.2 ± 17	91.9 ± 29.1	16	16/0	39 (23-70)		Unlabelled	0.15 mg/kg	Radioimmunoassay	Raubenheimer et al, 2006
Clearance ($l/hr/m^2$)	6.4 ± 1.3	2.7 ± 0.3	18	8/10	31 ± 3	Steady state	^{3}H	<0.037 µg/hr	Fluorometric method	Huther et al, 1970
	28.1 ± 3.4	9.5 ± 1.4	8	5/3	21-36	Steady state	^{3}H	ns*	ns	Messerli et al, 1976

Mean ± SEM or range
ns = not specified
*Tracer dose, 2.5 µCi/hr, mass not specified

1.3.3.5. Receptor affinities

Relative binding affinities of corticosterone and cortisol for GR and MR (section 1.1.4) vary according to the species studied, although MR is consistently reported as a high affinity receptor, with a Kd generally < 2.5 nM for both corticosterone and cortisol (Veldhuis et al, 1982; Krozowski et al, 1983; Sutanto et al, 1987; Reul et al, 1990). In humans, cloned MR has roughly equal affinity for corticosterone and cortisol (Kd ~ = 1.3 nM) (Arriza et al, 1987).

Limited study has been undertaken comparing the relative binding affinities of corticosterone and cortisol for the low affinity GR. In a comparative study in which human foetal lung tissue was studied, GR displayed enhanced binding affinity for the dominant glucocorticoid across a range of species, with a binding affinity for cortisol 1.4 x greater than that of corticosterone in humans (Giannopoulos et al, 1981). Study of mammalian cells transfected with human GR, however, revealed transactivation and binding was greater for corticosterone, by a factor of 1.5 and 2.0, respectively (Berger et al, 1992).

1.4. Trans-membrane trafficking of corticosterone and cortisol

A further factor that requires consideration when comparing the properties of the endogenous glucocorticoids corticosterone and cortisol is their ability to enter the cell to reach their principal site of action. Although glucocorticoids are highly lipophilic and likely to readily diffuse across the cell-membrane (Giorgi et al, 1981), accumulating evidence suggests active transport of glucocorticoids also occurs, due to the action of transmembrane proteins. This finding is analogous to thyroid hormones, for which importers have been discovered relatively recently (Heuer et al, 2009); however unlike thyroid hormone, active transporters for glucocorticoids appear to function exclusively as exporters. This phenomenon was first reported in the late 1960s (Gross et al, 1968), but little-studied until relatively recently. It remains unclear whether the process influences tissue sensitivity to glucocorticoids *in vivo*.

1.4.1. ATP-binding cassette (ABC) transporters

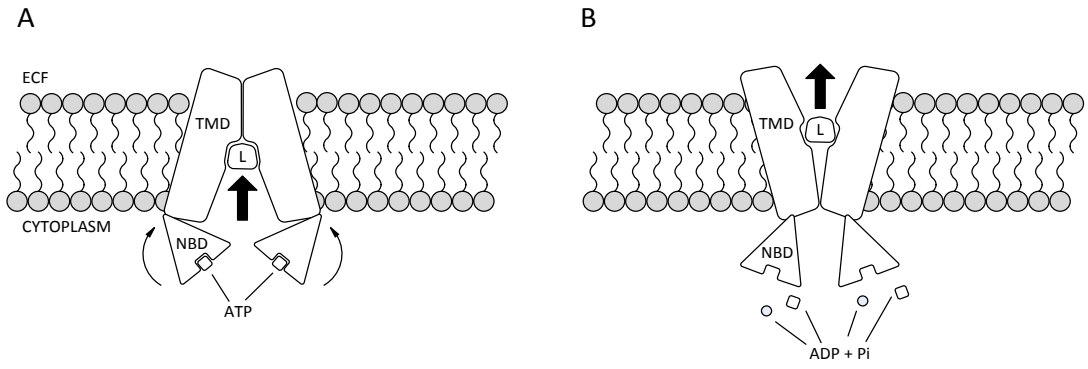
Transport of a substrate against a concentration gradient requires energy, which is derived either from coupling its transport with that of an energetically favourable substrate (co-transport), or from hydrolysis of adenosine triphosphate (ATP). ATP-binding cassette (ABC) transporters are a class of ATP-dependent membrane pumps present in all classes of organism (Higgins, 1992; Vasiliou et al, 2009). The 49 ABC proteins identified in humans have been classified in sub-families, designated A-G, according to phylogenetic analysis and amino acid sequence

(Vasiliou et al, 2009). They include both importers and exporters, and mostly act upon lipophilic substrates (Dean et al, 2001).

ABC proteins comprise two nucleotide-binding domains, which bind and hydrolyse ATP, and two trans-membrane domains, which facilitate substrate recognition and translocation across the lipid membrane. Analysis of their molecular architecture suggests the pumps operate according to an 'alternating access' model (Rees et al, 2009), with substrate binding resulting in ATP hydrolysis and a conformational change from an 'inward facing' to an 'outward facing' (in the case of an exporter). The relative binding affinities for substrate in each conformation are likely to determine whether the transporters function as exporters or importers (Locher et al, 2002).

Figure 1-8. Schematic of ABC transporter illustrating 'alternating access' mechanism of action

ABC proteins comprise two trans-membrane domains (TMDs) and two nucleotide-binding domains (NBDs). In the case of an exporter, ligand (L) binding in the 'inward facing' conformation (panel A) promotes hydrolysis of ATP, releasing energy required for a conformational change towards the 'outward facing' position (panel B). ECF = extracellular fluid.



1.4.2. ABCB1 preferentially exports cortisol over corticosterone from the central nervous system

ABCB1, also known as multidrug resistance 1 (MDR1) or P-glycoprotein (Pgp), was the first ABC transporter to be characterised, following the discovery of its role in anticancer drug resistance (Sugawara et al, 1988). Evidence from a number of sources suggests the protein also plays a role in the transport of glucocorticoids; and that this process is of particular relevance in the central nervous system (CNS), where it acts as a physiological barrier to the entry of cortisol, but not corticosterone.

1.4.2.1. Genetically modified mouse models

Reduced retention of cortisol in comparison to corticosterone in the rodent brain was first reported in the 1970s (McEwen et al, 1976; Pardridge et al, 1979). Several decades later, genetically modified mouse models began to provide insight into the role ABC transporters play in glucocorticoid trafficking in the CNS. In mice, two functional isoforms of ABCB1 exist, encoded by *Abcb1a* and *Abcb1b*, each with differing substrate affinities (Devault et al, 1990). In the most commonly used knockout mouse model, the *Abcb1a* gene is disrupted in isolation. Study of radiolabelled substrate has demonstrated increased CNS uptake of synthetic glucocorticoids (Meijer et al, 1998; Karssen et al, 2002) and cortisol, but not corticosterone in the *Abcb1a (-/-)* mouse (Karssen et al, 2001). This finding has not replicated by all investigators (Mason et al, 2012), perhaps related to the use of a different strain of mouse, or alternatively to the presence of the ABCB1B isoform in the single-knockout mouse. Findings from the double knockout mouse

[*Abcb1a/b(-/-)*], generally support the concept of ABCB1 specificity for cortisol, with little (Uhr et al, 2002) or no (Mason et al, 2008) effect on corticosterone transport.

It is likely that this process of active transport of glucocorticoids from the central nervous system occurs at the blood-brain barrier (BBB). The BBB is formed by the endothelial cells that line cerebral microvessels, with a number of adaptations not present in endothelium elsewhere, such as the presence of tight junctions, which means it forms a selective barrier to maintain neuronal function (Abbott et al, 2006). Knockout of *Abcb1a* in mice results in increased uptake of cortisol and synthetic glucocorticoids throughout the brain, but not in tissues unprotected by the BBB, including the pituitary gland (Meijer et al, 1998; Mason et al, 2008), liver, testes or intestine (Karssen et al, 2002). Localisation of ABCB1 to the luminal surface of brain capillaries confirms its presence at the BBB (Beaulieu et al, 1997), although it has also been detected in the murine parenchymal brain tissue including hippocampal neurons (Regina et al, 1998; Karssen et al, 2004).

1.4.2.2. *In vitro* studies

Data from *in vitro* studies complement the above findings from studies in transgenic mice. Study of various endogenous and exogenous glucocorticoids indicates the presence of a hydroxyl group at position 17, the sole difference between cortisol and corticosterone, is required for a glucocorticoid to act as an ABCB1 substrate (Bourgeois et al, 1993; Karssen et al, 2002). Glucocorticoid-induced transactivation in a mouse fibroblast cell line is increased following inhibition of endogenous ABCB1 in the presence of cortisol but not corticosterone (Webster et al, 2002).

Additionally, transfection of an epithelial cell line with human *ABCB1* results in polar transport of cortisol but not corticosterone, and inhibition of ABCB1 returns cortisol transport to the wild-type state (Karssen et al, 2001).

1.4.3. Role of ABCB1 in glucocorticoid transport in humans

ABCB1 is expressed in a limited number of tissues in humans. Many of these tissues can be grouped by their location in apical membranes of cells facing excretory compartments, including those in the small and large bowel, exocrine pancreas, hepatic canalicular and biliary epithelium, and brush border of the renal tubules (Thiebaut et al, 1987), suggesting ABCB1 may play a role in excretion of toxins in epithelial tissue. However, intestinal glucocorticoid transport may also be affected by ABCB1 activity, with increased expression of ABCB1 associated with a poorer response to glucocorticoid therapy in inflammatory bowel disease (Farrell et al, 2000).

In endothelial tissue, ABCB1 is generally not detected (Thiebaut et al, 1987; Sugawara et al, 1990). However, it is present in the adrenal gland, implying a possible role in glucocorticoid efflux, the blood-testes barrier, and at the BBB. ABCB1 is present in the human BBB from early gestation (Schumacher et al, 1997; Daood et al, 2008). In infancy and adulthood, the protein is present in capillary endothelial cells at BBB sites but not in vessels from other tissues, including the reproductive system (other than testes), stomach, colon, kidney, lungs and placenta (Cordon-Cardo et al, 1989; Jette et al, 1993). Evidence that the transporter is functional at the BBB in humans comes from the finding that administration of the

ABCB1 inhibitors cyclosporin and tariquidar increase penetration of the radiolabelled ABCB1 substrate ([^{11}C]-verapamil) in the brain and have no effect in areas outside the BBB (Eyal et al, 2010; Bauer et al, 2012).

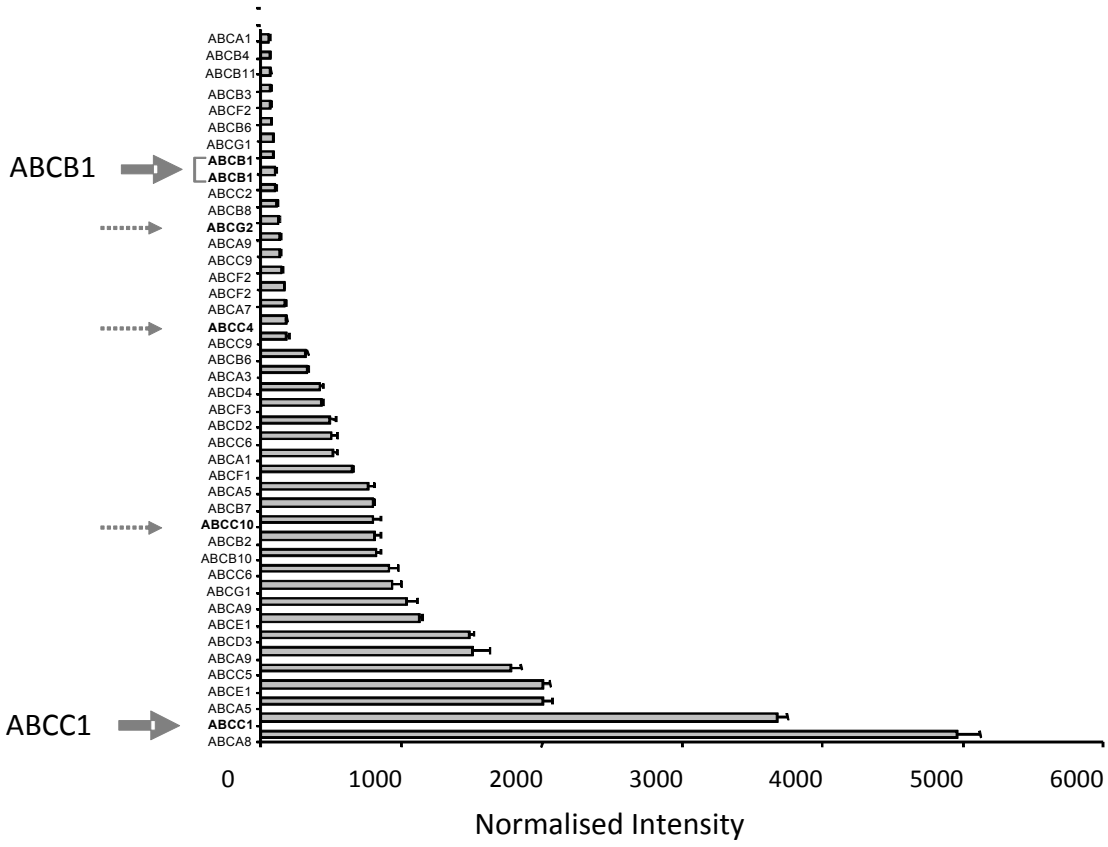
The finding that the BBB is a major site of ABCB1 activity, together with data from murine models and *in vitro* studies which suggest the protein exports cortisol but not corticosterone, have led to investigation of whether the corticosterone/cortisol ratio in the human CNS is higher than the corresponding plasma ratio. Data from non-human primates provided some support for this hypothesis, with evidence that corticosterone is preferentially retained over the 'dominant' glucocorticoid cortisol in the hippocampus (Gerlach et al, 1976). In human post-mortem brain tissue, Karssen and co-workers (Karssen et al, 2001) noted the corticosterone/cortisol ratio was approximately 5-fold greater than the plasma ratio in samples from age-matched controls. Similarly, Raubenheimer and co-workers (Raubenheimer et al, 2006), studying individuals undergoing lumbar puncture, found the corticosterone/cortisol ratio in cerebrospinal fluid (CSF) was 5.6 fold greater than the ratio from paired plasma samples. Although findings from the latter study reflect transport at the blood-CSF barrier, which does not constitute a blood-CNS barrier (Pardridge, 2011), ABCB1 is expressed at the blood-CSF barrier (Gazzin et al, 2008), meaning it is plausible that the transporter explains relative accumulation of corticosterone over cortisol in the CSF.

1.4.4. Selective export of corticosterone by an alternative transporter outside the CNS

Evidence suggests that while ABCB1 functions as a cortisol exporter in the CNS, an alternative transporter, ABCC1 (previously referred to as MRP1) exports corticosterone over cortisol in peripheral tissues. In contrast to their findings for ABCB1, Webster and colleagues (Webster et al, 2002) noted that glucocorticoid-induced transactivation in a mouse fibroblast cell line is increased following inhibition of endogenous ABCC1 in the presence of corticosterone but not cortisol. Unlike ABCB1, ABCC1 is not expressed in the human BBB (Daood et al, 2008) but is expressed in adipose tissue (Figure 1-9, supervisor's unpublished data).

Figure 1-9. Transcripts for ATP-binding cassette transporters in human subcutaneous adipose tissue

Transcript microarrays in subcutaneous adipose tissue in 10 healthy men. Relative intensity is shown for transcripts for members of the ABC transporter family, ranked by intensity. Note that ABCB1, the cortisol exporter in CNS, is not significantly expressed in adipose tissue, whereas ABCC1, the corticosterone exporter, is highly expressed in adipose tissue. The dotted arrows indicate other ABC transporters that are thought to transport steroids.



1.4.5. Implications of tissue-specific ABC-transporter-mediated glucocorticoid trafficking

A number of mechanisms exist which account for varying sensitivity to glucocorticoid action between tissues (section 1.1.4). Little is known about the extent to which the differences in the function and localisation of ABC transporters

discussed above determine glucocorticoid access to their cytosolic receptors and thus influence tissue sensitivity to their action.

1.4.5.1. The HPA axis

Few robust markers for glucocorticoid action within the CNS exist, but measurement of activity of the HPA axis reflects CNS glucocorticoid action in the hypothalamus and hippocampus (in addition to that in the pituitary gland, outside the CNS). Activity of ABCB1 at the BBB would be expected to reduce cortisol-mediated feedback in the hypothalamus and hippocampus and result in activation of the HPA axis. Because of the differential expression of high and low affinity corticosteroid receptors in feedback centres protected or unprotected by the BBB, respectively (Reul et al, 1985; de Kloet et al, 1998), the magnitude of such effects may be dependent upon prevailing plasma glucocorticoid concentrations.

In the [*Abcb1a/b (-/-)*] mouse, plasma corticosterone is reduced (Muller et al, 2003; Yau et al, 2007) alongside plasma ACTH concentration and hypothalamic CRH mRNA (Muller et al, 2003), consistent with at least a small effect of ABCB1 on corticosterone transport as previously described (Uhr et al, 2002). ABCB1-mediated cortisol transport is more difficult to assess in corticosterone-predominant species, and it is not known from transgenic mouse studies whether preferential transport of cortisol over corticosterone occurs. However, some insight into ABCB1 transport in cortisol-predominant species can be gained from the study of Collie dogs, of which approximately one-third to one-half are homozygous for a deletion mutation in *ABCB1* associated with reduced transporter function (Mealey et al, 2002; Hugnet et

al, 2004), accompanied by lower basal and stimulated cortisol concentrations and enhanced ACTH suppression to dexamethasone (Mealey et al, 2007). Similarly, in humans, a polymorphism in *ABCB1*, 3435 C>T (rs1045642), which reduces ABCB1 mRNA stability (Wang et al, 2005) has been associated with reduced serum cortisol concentration (Nakamura et al, 2009).

Preferential transport of cortisol would mean that corticosterone might be expected to 'bypass' the effect of ABCB1 at the BBB and exert a greater effect on the HPA axis than would be predicted by its low plasma concentration relative to cortisol. Raubenheimer and colleagues (Raubenheimer et al, 2006) assessed this in healthy human volunteers by measurement of ACTH following an intravenous bolus of cortisol or corticosterone, after suppression of endogenous glucocorticoids with metyrapone. Although no significant difference in ACTH suppression between the two steroids was found, direct comparison was difficult due to the administration of bolus doses of glucocorticoids, which meant subjects did not attain equivalent plasma glucocorticoid concentrations. Additionally, because metyrapone induces accumulation of intermediate steroids such as 11-deoxycorticosterone (Steiner et al, 1994), which are potent MR agonists (Arriza et al, 1987), its effects on the HPA axis are uncertain and potentially confounding.

1.4.5.2. Metabolic syndrome

In metabolic syndrome, resistance to the negative feedback actions of glucocorticoids is seen, for reasons that have not been fully characterised (section 1.2.7). Despite this, phenotypic changes consistent with chronic glucocorticoid

excess continue to be displayed in peripheral tissues. In principle, tissue-specific expression of ABC transporters may go some way to explain this apparent paradox. Activity of ABCB1 at the BBB may limit access of cortisol to the hypothalamus and hippocampus. Differential substrate affinities may mean that this physiological barrier is not present for corticosterone. A relatively low plasma corticosterone may therefore drive activation of the HPA axis, increasing risk of metabolic syndrome. Importantly, the presence of alternative transporters in peripheral tissues (eg. ABCC1 in adipose), would result in ongoing sensitivity to cortisol excess while simultaneously limiting their response to variations in plasma corticosterone.

1.4.5.3. Adrenal insufficiency

Disproportionate CNS sensitivity to corticosterone over cortisol may also be of clinical relevance in adrenal insufficiency. Adrenal insufficiency is categorised into two types, according to the site of pathology. In primary adrenal insufficiency (Addison's disease), deficiency of glucocorticoids and mineralocorticoids is caused by a disease process directly affected the adrenal gland: most commonly autoimmune disease, but also including infective, malignant or infiltrative pathology (Charmandari et al, 2014). A further form of primary adrenal insufficiency, considered a separate entity to Addison's disease but sharing many similarities, is congenital adrenal hyperplasia (CAH). CAH encompasses a wide range of clinical conditions, each resulting from a congenital defect in adrenal steroidogenesis (Speiser et al, 2003). A shared feature of all forms of CAH, however, is activation of the HPA axis due to glucocorticoid deficiency, often causing clinically problematic adrenal androgen excess. Secondary adrenal insufficiency is characterised by ACTH

insufficiency, most commonly due to suppression of the HPA axis following chronic glucocorticoid therapy, or structural disease of the hypothalamus and/or pituitary.

Individuals with adrenal insufficiency are unable to mount an adequate stress response to illness or injury, and in these situations a potentially fatal 'adrenal crisis' results from inadequate glucocorticoid and mineralocorticoid replacement. Untreated adrenal insufficiency is also accompanied by a number of non-specific symptoms, as recognised by Thomas Addison in 1855 in his original description of the condition: "anaemia, general languor and debility, remarkable weakness of the heart's action, irritability of the stomach, and a peculiar change of colour in the skin." (Addison, 1855). Individuals with Addison's disease are generally treated with 15-25 mg cortisol (hydrocortisone)/day (Hahner et al, 2009), and frequently develop non-specific symptoms at lower doses, even though in healthy individuals, normal cortisol output is as low as 10 mg/day (Esteban et al, 1991). Despite current treatment strategies, individuals with Addison's disease are at increased risk of mood disorders (Thomsen et al, 2006) and report impaired subjective health status (Lovas et al, 2002), even after exclusion of those with co-morbidities (Hahner et al, 2007). Non-specific CNS symptoms may reflect deficient or excess glucocorticoid action in the brain, or be related to physical manifestations of adrenal insufficiency and its treatment. However, evidence that current replacement strategies inadequately replace glucocorticoids in the CNS comes from a UK survey of treated CAH patients (Arlt et al, 2010), in whom androgen levels are generally poorly controlled, reflecting ACTH excess and inadequate glucocorticoid signalling in the feedback centres of the CNS.

In the peripheral tissues, adequate biomarkers of glucocorticoid action do not exist. However, in the above survey, patients were noted to have an adverse metabolic profile, consistent with peripheral glucocorticoid excess. This may also underlie reduced bone mineral density in Addison's disease (Lovas et al, 2009; Bjornsdottir et al, 2011). Moreover, in patients with primary or secondary adrenal insufficiency, cardiovascular mortality is increased (Bergthorsdottir et al, 2006; Bensing et al, 2008), and in patients with secondary adrenal insufficiency, replacement with >20 mg cortisol/day is associated with features of metabolic syndrome (Filipsson et al, 2006).

The limitations of currently available therapies for adrenal insufficiency are widely acknowledged (Debono et al, 2009; Forss et al, 2012; Grossman et al, 2013). It is possible that this is due to difficulty mimicking the normal ultradian and circadian profiles of plasma glucocorticoid concentrations due to the pharmacokinetic properties of oral glucocorticoids in use. Varying the dosage and timing of these preparations has little effect on subjective health status (Bleicken et al, 2010); instead focus has shifted to novel therapies aimed at achieving a more physiological circadian profile, including subcutaneous hydrocortisone infusion (Oksnes et al, 2014), modified-release (Verma et al, 2010) and dual-release (Johannsson et al, 2009) oral preparations.

In common with preparations currently used in clinical practice, novel therapies have been developed under the assumption that a single glucocorticoid is of importance. Limited ability of cortisol and synthetic glucocorticoids to penetrate the CNS due to action of ABCB1 may account for central symptoms of glucocorticoid deficiency in

treated hypoadrenal patients who continue to display features of glucocorticoid sensitivity in peripheral tissues. Replacing deficient corticosterone may optimise CNS glucocorticoid action in this patient group and enable a reduction in cortisol dose to better represent normal physiology.

1.5. Hypotheses

- Physiological negative feedback suppression of ACTH is disproportionately sensitive to corticosterone, while adipose tissue is disproportionately sensitive to cortisol.
- In metabolic syndrome, negative feedback suppression of the HPA axis is pathologically impaired as a result of low plasma corticosterone.
- In adrenal insufficiency, replacement of corticosterone in addition to cortisol optimises glucocorticoid activity in the CNS.

1.6. Aims

1. To validate an assay for plasma corticosterone using liquid chromatography-tandem mass spectrometry (LC-MS/MS)
2. To validate the stable isotope 2,2,4,6,6,17α,21,21-[^{2}H]$_{8}$-corticosterone as a tracer for corticosterone
3. To determine the production rates and clearance of corticosterone in healthy volunteers using the principle of isotope dilution
4. To compare cortisol and corticosterone effects on the HPA axis and adipose tissue *in vivo*
5. To test for 'relative corticosterone deficiency' in metabolic syndrome

Chapter 2: Materials and methods

2.1. Equipment

a) Balance

Mettler Toledo MT5 microbalance (Mettler Instrumente Ag, Zürich, Switzerland)

b) Centrifuges

- Hepatic cytosol preparation (section 4.2.1): Optima TLX Ultracentrifuge, (Beckman Coulter, High Wycombe, UK)
- D8-corticosterone LC-MS/MS (Chapter 5): Eppendorf centrifuge 5810R (Cambridge, UK)
- Clinical sample processing (Chapter 6): Sigma 4K15 centrifuge (Münich, Germany)

c) Homogeniser

Pro200 homogeniser (Pro scientific Inc, Oxford, CT)

d) Microplate shaker

iEMS Incubator/shake (Thermo Fisher Scientific Inc, Waltham, MA)

e) Microplate washer

Anthos aw1 automatic plate washer (Anthos Labtec systems, Salzburg, Austria)

f) Microplate reader

Optimax tuneable microplate reader (Molecular Devices, Sunnyvale, Ca)

g) Nitrogen dry-block

Dri-Block® DB3A sample concentrator (Techne, Staffordshire, UK)

h) Ovens

- Memmert D88 oven (Memmert, Nürnberg, Germany)

- Hybaid mini oven (VWR International, Radnor, PA)

i) pH meter

Jenway 3510 pH meter (Bibby Scientific Ltd, Stone, UK)

j) Shaking water bath

OLS 200 Shaking water bath (Grant Instruments, Cambridge, UK)

k) Vortex

Rotamixer (Hook and Tucker Instruments, Longfield, UK)

2.2. Materials

All reagents were obtained from Sigma Aldrich (St. Louis, MO) or VWR International (Radnor, PA) unless otherwise stated. All solvents were glass distilled HPLC grade from Thermo Fisher Scientific Inc (Waltham, MA).

Room temperature is defined as a temperature between 18 and 22 °C.

2.2.1. Steroids

2.2.1.1. Unlabelled steroids

Corticosterone, epi-corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, cortisol, progesterone and dehydroepiandrosterone sulphate (DHEAS). Each of these steroids were weighed and dissolved in methanol (final concentration 1 mg/ml) and stored at -20 °C.

2.2.1.2. Deuterium labelled steroids

Deuterium labelled steroids were obtained from Cambridge Isotope Laboratories (Andover MA). Chemical and isotopic purity were assessed by the manufacturer. For *in vitro* studies, these were weighed and dissolved in methanol (final concentration 1 mg/ml) and stored at -20 °C. Preparation of the steroids for their use as stable isotope tracers in clinical studies is described in Chapter 6.

a) D8-corticosterone

   Corticosterone (2, 2, 4, 6, 6, 17α, 21, 21-[^{2}H]$_{8}$, 98%)

b) D4-cortisol

Cortisol (9, 11, 12, 12-[^{2}H]$_{4}$, 98%)

2.2.1.3. Tritium labelled steroids

Tritium labelled steroids were obtained at the following concentrations (in ethanol) and stored at -20 °C.

a) Corticosterone [1, 2, 6, 7-^{3}H(N)]

1 mCi/ml, 78.1 Ci/mmol

b) [^{3}H]$_{4}$-11-dehydrocorticosterone

1 mCi/ml, 78.1 Ci/mmol

(a) and (b) were obtained from American Radiolabelled Chemicals Inc (St Louis, MO).

c) Cortisol [1, 2, 6, 7-^{3}H(N)]

1 mCi/ml, 95.4 Ci/mmol, Perkin Elmer, Boston, MA

d) Cortisone [1, 2, 6, 7-^{3}H(N)]

1 mCi/ml, 51.0 Ci/mmol, Amersham Radiochemicals, Cardiff, UK

2.2.1.4. ^{125}I labelled corticosterone conjugate

MP Biomedicals, Cambridge, UK (supplied at 1500-200 μCi/μg). Diluted according to manufacturer's instructions in borate buffer.

2.2.2. Commonly used buffers

a) Potassium phosphate buffer

50 mM potassium dihydrogen phosphate (KH_2PO_4), 1 mM ethylenediaminetetraacetate (EDTA) dissolved in distilled water, adjusted to pH 7.4 using potassium hydroxide (1 M), and stored at 4 ° C.

b) Borate buffer

Boric acid (8.25 g), sodium hydroxide (2.7 g) was dissolved in distilled water (1 l) adjusted to pH 7.5. Bovine serum albumin (5 g; fraction V) was added and buffer stored at -20 °C in aliquots of 50 ml.

c) Sodium acetate buffer

Sodium acetate (0.2 M) dissolved in distilled water, adjusted to pH 4.6 using glacial acetic acid, and stored at 4 °C.

2.2.3. Drugs and reagents for corticosterone radioimmunoassay

a) Scintillation proximity assay (SPA) beads

Anti-sheep (yttrium silicate) 500 mg, Perkin Elmer (Buckinghamshire, UK) were dissolved in borate buffer (100 ml), stored (-20 °C) in aliquots (5 ml).

b) Anti-corticosterone antibody

This was a kind gift from Dr Chris Kenyon (Edinburgh, UK) and was raised in sheep. Antibody was diluted (1 in 800) in borate buffer and stored (-20 °C) in aliquots (150 µl). After defrosting, it was diluted (1 in 100) in borate buffer to achieve a working dilution (1 in 80 000).

2.2.4. Drugs and reagents for *in vitro* assays

Reagents for *in vitro* assays were obtained from Sigma Aldrich (St. Louis, MO).

a) Glucose-6-phosphate dehydrogenase (G6P-DH), from baker's yeast (*S.cerevisiae*).

   Supplied as crystalline suspension in 3.2 M $(NH_4)_2SO_4$ solution, pH 7.0, containing 250 units. Diluted in potassium phosphate buffer (250 µl) and stored (-4 °C) in aliquots (10-20 units).

b) D-glucose-6-phosphate dipotassium salt hydrate (G6P). Stored at -20 °C.

c) β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH). Stored at -20 °C.

2.2.5. Drugs and reagents for cell culture

All cell culture reagents were obtained from Lonza (Slough, UK).

a) Dulbecco's Modified Eagle's Medium

   Containing heat-inactivated fetal calf serum (FCS) (10% *v/v*), glucose 4.5 g/l, glutamine (2 mM) and penicillin (1% *w/v*)/streptomycin (1% *w/v*)

b) Phosphate-buffered saline (PBS)

   Dulbecco's PBS with calcium chloride dihydrate (0.884 mM) and magnesium chloride hexahydrate (0.492 mM)

c) Trypsin /EDTA

   Trypsin 1:250 (500 mg/l), tetrasodium EDTA (200 mg/l)

d) Poly-D-lysine (PDL) hydrobromide (0.1 mg/ml).

   This was stored at -20 °C in aliquots of 5 ml.

e) Charcoal stripped serum

Foetal calf serum (FCS; 500 ml) and dextran coated charcoal (5 g) were mixed (~16 hours, 4 °C). The solution was filtered through a 250 μm filter funnel and separated into aliquots and stored at -20 °C until use.

2.2.6. Human serum and hepatic cytosol

a) Human serum, steroid stripped

This was obtained from TCS Biosciences (Buckingham, UK) and comprises pooled sterile filtered serum derived from plasma. Serum was divided into aliquots (5 ml) and stored (-20 °C).

b) Human hepatic cytosol

Pooled cytosol (~20 mg per ml) prepared from adult human donors. Storage (-80 °C) was in aliquots (100 μl) of undiluted cytosol which were thawed rapidly in a water bath (37 °C) then kept on ice before use.

2.2.7. Derivatisation agents for GC-MS

a) Mobile phase: 98 % cyclohexane (*v/v*), 1 % hexamethyldisilazane (*v/v*), 1 % pyridine (*v/v*)

b) Lipidex 5000 (Packard, Pangbourne, UK): Lipidex 5000 was suspended in methanol, and filtered. Methanol was discarded and beads re-suspended in mobile phase (a) (200 ml) and stored, protected from light, at room temperature.

c) Methoxyamine in pyridine: methoxyamine hydrochloride in pyridine (2 % *w/v*)

d) Trimethylsilylimidazole (TMSI): undiluted

2.2.8. Solutions and solvents for clinical studies

a) Saline (NaCl)

   Sodium chloride (0.9% *w/v*) (Baxter, Newbury, UK), containing 77 mmol/500 ml (154 mM) sodium and 77 mmol/500 ml (154 mM) chloride

b) Dehydrated alcohol BP

   Ethanol (100% *v/v*) (Martindale, Brentwood, UK)

c) DEPC (Diethylpyrocarbonate) water

   DEPC (10 drops) was added to distilled water (1 l). This was mixed and allowed to stand (room temperature) for 24 hours before autoclaving. Storage was at room temperature.

2.3. General Laboratory Methods

2.3.1. Quantification of plasma corticosterone by radioimmunoassay (RIA)

2.3.1.1. Method

Plasma corticosterone levels were measured using an in-house RIA (Al-Dujaili et al, 1981) modified for microtiter plate scintillation proximity assay (SPA). Plasma was diluted 1 in 10 in borate buffer and stored (-20 °C) prior to assay. Standard solutions (0 – 80 nM) were prepared in borate buffer. Samples, standards, borate buffer and SPA beads were defrosted on wet ice and mixed. Sample or standard (10 µl) was pipetted in duplicate into a 96 well plate (Thermo Fisher Scientific Inc, Waltham, MA). Samples were heat denatured (70 °C, 30 mins) to dissociate glucocorticoids from CBG, and then allowed to cool to room temperature. Sheep anti-corticosterone antibody (25 µl) and SPA beads (25 µl) were added under constant magnetic stirring. Corticosterone [^{125}I] conjugate (25 µl) was added and plates were sealed, mixed and incubated (10 hours, room temperature). Radioactive emissions were counted by liquid scintillation (Wallac al1450 Microbeta Plus Liquid Scintillation Counter, PerkinElmer, Waltham, MA).

2.3.1.2. Data Analysis

Radioactive counts from standards were fitted to a 2 parameter logistic inverse sigmoid curve (Assay Zap, Version 3.1, Biosoft, Cambridge, UK). To assess cross-reactivity, IC_{50} was calculated by determining a four-parameter dose-response curve (log (inhibitor) vs. Response – variable slope) using a least squares (ordinary)

fit (GraphPad Prism, Version 5.01, GraphPad Software Inc, La Jolla CA). The best-fit values of log IC_{50} were compared using the extra sum-of-squares F test. Data were accepted if the co-efficient of variation (CV) between duplicates was less than 15 %.

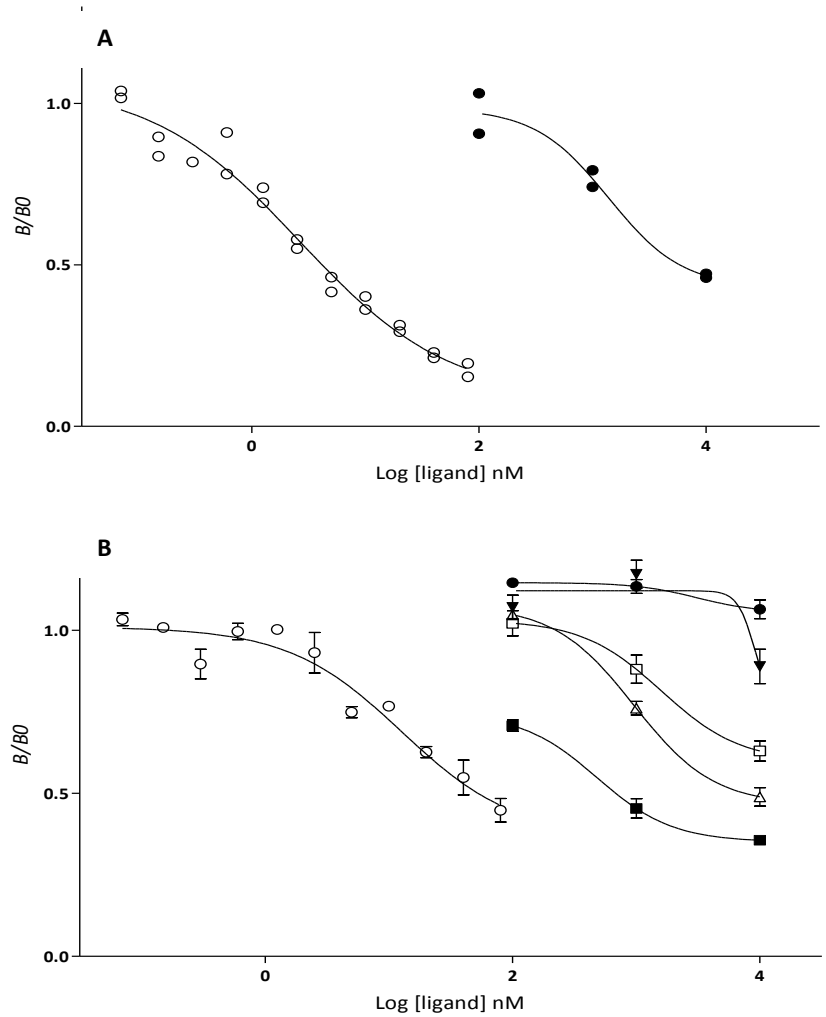
2.3.1.3. Cross-reactivity

Cross reactivity of the assay was determined for steroids chosen on the basis of a similar molecular structure to corticosterone (eg. 11-deoxycorticosterone), relative abundance in human plasma (eg. DHEAS), or presence of exogenous steroid in plasma samples (dexamethasone; section 3.2.1.3). The assay was performed for corticosterone solutions of known concentration (0-80 nM) to create a standard curve, which was compared to the standard curve produced from cross-reactants at concentrations of 100 nM, 1 μM and 10 μM (Figure 2-1). Cross-reactivity was calculated by determining the ratio of IC-50 for each cross-reactant over the corresponding IC-50 for corticosterone.

Cross-reactivity for all glucocorticoids (Figure 2-1) was less than 1 %, with the exception of 11-deoxycorticosterone (2.6 %) and dehydrocorticosterone (1.3 %). Importantly, both compounds normally circulate at less than 1 nM (Morris et al, 1953; Greenspan et al, 2001); indicating cross-reactivity is unlikely to impact significantly on estimates of serum corticosterone produced in the assay.

Figure 2-1. Corticosterone radioimmunoassay: cross reactivity

A: standard curve illustrating cross-reactivity of cortisol (closed circles) in radioimmunoassay for corticosterone (open circles) in duplicate. B/B_0 = antibody bound radioactivity B adjusted for binding at zero standard B_0. IC-50 = half maximal inhibitory concentration. B: Cross-reactivity for the alternative ligands: DHEAS (closed circles); testosterone (closed triangles); progesterone (open squares); 11-dehydrocorticosterone (open triangles); 11-deoxycorticosterone (closed squares). N = 3, mean ± SEM.



Corticosterone			Cross-reactant			Cross-reactivity
IC-50 (nM)	95% CI	r^2		IC-50 (nM)	r^2	
2.51	1.43-4.40	0.96	Cortisol	1387	0.97	0.18 %
12.72	4.15 -39.0	0.90	11-deoxycorticosterone	481	0.96	2.64 %
			11-dehydrocorticosterone	957	0.98	1.33 %
			Testosterone	9096	0.68	0.14 %
			Progesterone	1600	0.90	0.80 %
			DHEAS	3082	0.54	0.41 %

2.3.2. Quantification of plasma ACTH by enzyme-linked immunosorbent assay (ELISA)

Plasma ACTH was measured using a two-site ELISA (IBL International, Hamburg, Germany). All reagents were stored at 2-8 °C unless otherwise stated, and allowed to come to room temperature before each assay. Assay standards and controls were reconstituted by adding deionised water (2 ml) to each standard and control bottle. Reconstituted standards and controls were stored (-10 °C) for up to 3 freeze-thaw cycles. Antibody to the N-terminal of human ACTH conjugated to horseradish peroxidase (HRP) was freshly diluted 1: 21 in supplied diluent for each assay. Wash buffer concentrate (30 ml) was diluted by adding deionised water (870 ml) and stored (room temperature).

For each patient, samples from both study visits were assayed together. Assay standards (0-89 pmol/l, controls and samples (200 µl) were added in duplicate to a 96-well microtiter plate coated with antibody to the C-terminal of human ACTH. Diluted HRP conjugated antibody (25 µl) was added, forming a "sandwich" of solid-phase polyclonal antibody-human ACTH-HRP conjugated monoclonal antibody. Plates were sealed, protected from light and incubated on a plate shaker (400 rpm, room temperature, 4 hours). Unbound antibodies and buffer matrix were then removed by washing 5 times with working wash solution (350 µl). For the detection of the immunocomplex, tetramethylbenzidine (TMB) substrate solution (200 µl) was added to each well, plates were protected from light and incubated (static, room-temperature, 20 minutes). The HRP/TMB reaction was terminated with TMB stop solution (50 µl), plates were gently mixed and absorbance was read (450

nm; reference filter 650 nm) in a spectrophotometric microtiter plate reader. A standard curve was generated by plotting absorbance against plasma ACTH concentration for each standard by fitting to a 4-parameter curve (SoftMax Pro, Sunnyvale, Ca).

Intra-assay CV, as assessed by measurement of ACTH concentration in supplied controls in n = 8 assays, using mean results of duplicates in each assay, was 2.6 % ± 0.8 % and 4.2 % ± 1.1 % at 31.7 and 322 pg/ml respectively (n = 8). Inter-assay CV was 12.8 % at 7.0 pmol/L and 6.1 % at 70.9 pmol/L. Mean accuracy at 70.9 pmol/L was 97.3 % (range 88.2 % - 105.7 %).

2.3.3. Quantification of serum insulin by ELISA

Serum insulin was measured using a two-site ELISA (DRG Diagnostics, Marburg, Germany). All reagents were stored at 2-8 °C and allowed to come to room temperature before each assay. Peroxidase conjugated mouse monoclonal anti-insulin antibody was freshly diluted 1: 11 in supplied diluent for each assay. Wash buffer concentrate (50 ml) was diluted by adding deionised water (1000 ml). Assay standards containing recombinant human insulin (0 - 20 mU/l) and samples (25 µl) were added in singlicate to wells of a microtiter plate coated with mouse monoclonal anti-insulin antibody. Diluted peroxidase conjugated antibody (100 µl) was added. Plates were sealed, protected from light and incubated on a plate shaker (900 rpm, room temperature, 1 hour). Unbound antibodies and buffer matrix were then removed by washing 5 times with working wash solution (350 µl). TMB substrate solution (200 µl) was added to each well, and plates were incubated (static,

room-temperature, 30 minutes). TMB stop solution (0.5 M H_2SO_4, 50 µl) was added, plates were gently mixed and absorbance read (450 nm) in a spectrophotometric microtiter plate reader. A standard curve was generated by plotting absorbance against serum insulin concentration for each standard using cubic spline regression (SoftMax Pro, Sunnyvale, Ca).

2.3.4. Quantification of serum glucose by colorimetric assay

Serum glucose was measured by colorimetric assay (Cayman Chemical, Ann Arbor, MI). Sodium phosphate assay buffer concentrate (250 mM, pH 7.2) was diluted 1: 5 in deionised water and stored (2-8 °C). Remaining assay reagents were stored at -20 °C. Reagents and samples were placed on ice during assay preparation. Serum samples were diluted 1:5 in assay buffer and mixed. Glucose standards (0 - 1.39 mM) were prepared by dilution of glucose stock (5.55 mM) in assay buffer. Glucose colorimetric enzyme mixture was reconstituted with 6 ml assay buffer and mixed. Assay buffer (85 µl) was added to each of the wells of a 96-well microtiter plate. Standards and diluted samples (15 µl) were added, followed by working colorimetric enzyme mixture (100 µl), containing glucose oxidase, which undergoes flavin adenine dinucleotide (FAD) dependent reduction when incubated with glucose. The plate was covered and incubated (room temperature, 10 mins). Regeneration of glucose oxidase by oxidation results in the formation of hydrogen peroxide, which subsequently reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine, catalysed by HRP, to generate a pink dye. After gentle mixing, absorbance was read (500 nm) in a spectrophotometric microtiter plate reader and serum glucose was quantified from a standard curve generated by linear regression

analysis of absorbance (adjusted for zero standard) plotted against glucose concentration in standards.

2.3.5. Quantification of plasma non-esterified fatty acids by colorimetric assay

Plasma non-esterified fatty acids (NEFAs) were quantified by colorimetric assay (Zen-Bio, NC). Standards (0 - 333 μM, 50 μl), prepared by serial dilution in supplied dilution buffer, were added to the wells of a 96-well plate. Plasma (5 μl) was added to empty wells and diluted by adding dilution buffer (50 μl). Reconstituted reagent A (100 μl), containing acyl-CoA synthetase, was added to each well. After gently mixing, the plate was incubated (37 °C, 10 minutes) to produce fatty acyl-CoA thiol esters, which react with oxygen in the presence of acyl-CoA oxidase to produce hydrogen peroxide. Reconstituted reagent B (50 μl), containing peroxidase, was then added to each well, allowing the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple product. After gently mixing, the plate was incubated (37 °C, 10 minutes) then allowed to equilibrate (room temperature, 5 minutes). The optical density of each well was measured (550 nm) and plasma NEFA concentration calculated from a standard curve generated by linear regression analysis of absorbance (adjusted for zero standard) plotted against NEFA concentration in standards.

2.3.6. Quantification of plasma glycerol by colorimetric assay

Plasma glycerol was quantified by colorimetric detection of the product of a coupled enzyme reaction involving glycerol kinase and glycerol phosphate oxidase

(Sigma-Aldrich; St. Louis, MO). Reagents (stored at -20 °C) were allowed to come to room temperature, with the exception of enzyme mix, which was kept on ice until required. Standards (0 - 1.0 mM; in deionised water) and samples (10 µl) were added to the wells of a 96-well plate. Master reaction mix, containing assay buffer, enzyme mix, ATP, and dye reagent (100: 2: 1:1 *v/v*) was added (100 µl) to each well. Plates were incubated, protected from light, in a microplate shaker (37 °C; 400 rpm; 20 minutes). Absorbance was measured (570 nm) and plasma glycerol concentration calculated from a standard curve generated by linear regression analysis of absorbance (adjusted for zero standard) plotted against glycerol concentration in standards.

2.3.7. Quantitation of urinary corticosterone metabolites by gas chromatography/tandem mass spectrometry (GC-MS/MS)

2.3.7.1. Extraction of steroids from urine or aqueous standards

Steroids in urine (20 ml) or water (containing known amounts of steroids), both containing internal standard (5 µg epi-cortisol, 30 µg epi-tetrahydrocortisol, 30 µg epi-tetrahydrocorticosterone), were retained on conditioned Sep-Pak C18 columns (section 4.2.2.3) and steroids were eluted in methanol (2 ml). Glucocorticoid conjugates were hydrolysed by incubating (37 °C; 48 hours) with β-glucuronidase (Helix pomatia, Type H-2, 85 000 units/ml; 100 µl) in sodium acetate buffer (2 ml). Steroids were retained from hydrolysates by passing through conditioned Sep-Pak C18 columns as above, and the eluate reduced to dryness under OFN (60 ºC). Steroidal extracts were re-constituted in water (200 µl), re-extracted with ethyl

acetate (2 ml) and the organic layer dried (OFN, 60 ºC) in glass Reacti-vials (Thermo-Scientific Inc, Waltham, MA).

2.3.7.2. Derivatisation

Preparation of Lipidex 5000 occlusion columns

Each column was prepared by plugging a 150 mm glass Pasteur pipette with a silanised glass wool to create a column depth of ~ = 1 inch. Lipidex 5000 (2 ml) in mobile phase was added to each column, shaking regularly to prevent settling, and excess mobile phase allowed to elute. The trapped matrix was washed with mobile phase (3 x 1 ml)

Derivatisation conditions

The following method was used to form methoxime-trimethylsilyl (MO-TMSi) derivatives (Best et al, 1997): standards/dried organic extracts were dissolved in methoxyamine hydrochloride (50 µl, 2 % *w/v* in pyridine), vortexed, capped and incubated (30 minutes, 60 °C). After drying (60 °C OFN), trimethylsilylimidazole was added, and vials were capped, vortexed and incubated (2 hours, 100 °C). The sample was re-suspended in mobile phase (1 ml) before being passed through a Lipidex 5000 column. Reacti-vials were washed with mobile phase (2 x 1 ml), which was also applied to the column. The eluate was collected in 3.5 ml disposable glass vials, reduced to dryness (OFN, 60 °C), re-suspended in decane (100-200 µl), transferred to chromatography vials and stored (-20 °C) for analysis by GC-MS/MS.

2.3.7.3. Analysis by GC-MS/MS

Steroids in derivatised extracts were quantified using a TSQ Quantum XLS Ultra™ triple quadrupole mass spectrometer connected to a TRACE Ultra Gas Chromatograph, (Thermo Scientific Inc, Waltham, MA).

Gas Chromatography

Steroids in decane were injected (1 µl; room temperature) using a TriPlus autosampler (Thermo Scientific Inc) and vaporized in mobile phase (helium gas) heated (270 °C) and maintained at a constant flow rate (2 ml/min). For final quantitative analysis (Table 2-1), chromatographic separation was achieved using a Zebron ZB-HT Inferno column (Phenomenex, Torrance, Ca) measuring 30 m x 0.25 mm (internal diameter) x 0.25 µm (film thickness). After injection by splitless programmable temperature vaporization (PTV), the column was heated by 30 °C/min, maintained at 200 °C for 1 minute, and finally heated by 5 °C/min until target temperature was reached (300 °C; held for 3 minutes). Total chromatographic run time was 27.7 mins. Practice assays and full scan analyses (Figure 2-2) were performed under the same conditions using a J&W DB-17ms column (30 m x 0.25 mm x 0.35 µm; Agilent Technologies, Santa Clara, CA).

Tandem mass spectrometry

Positively charged ions were formed by electron ionisation at 280 °C in a Durabrite Iris source (electron energy 70 eV, emission current 50 µA). Quantitative analysis was performed in multiple reaction monitoring (MRM) mode (cycle time 0.3 s) using

collision induced dissociation (argon gas; 1.0 mTorr). Collision energies and MRM transitions are listed in Table 2-1.

Where an MS/MS method had not previously been established (17-deoxycortol and 17-deoxycortolone), derivatised standards were analysed in full scan (Q1) mode (representative data for 17-deoxycortol are shown in Figure 2-2), followed by a product ion scan to determine optimal MRM transitions and collision energies.

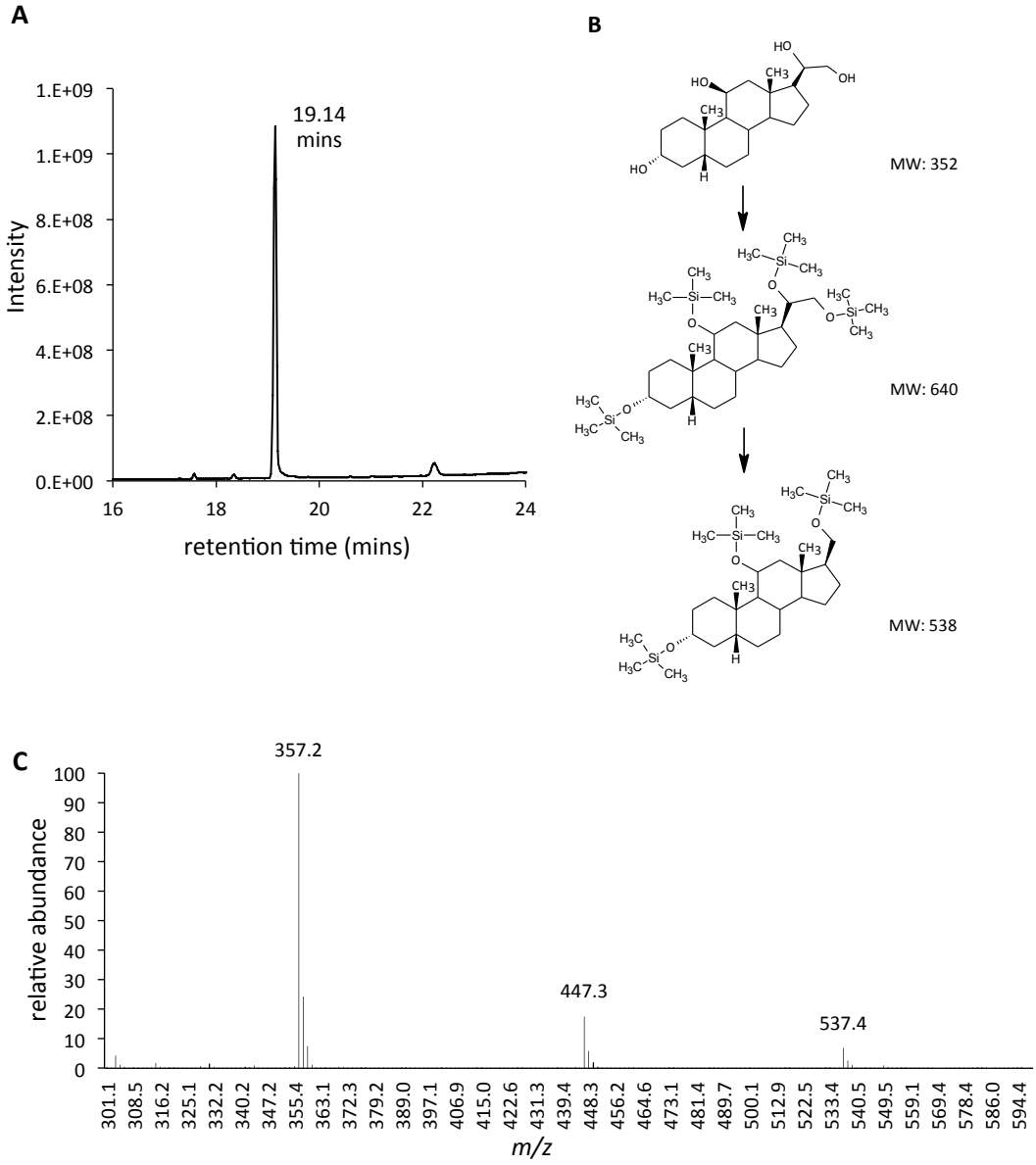
Table 2-1. Retention times, collision energies and mass/charge (*m/z*) ratios used for the quantitation of urinary steroids by GC-MS/MS

			Retention time (mins)	Collision energy (V)	*m/z*	
					Precursor ion	Product ion
17-deoxy steroids	11-Dehydrocorticosterone (A)		25.7	10	474.3	443.2
	Corticosterone (B)		24.5	15	548.3	517.3
	Tetrahydro-11-dehydrocorticosterone (THA)		21.5	15	521.5	400.4
	Tetrahydrocorticosterone (THB)	5β-THB	20.0	15	474.2	384.2
		5α-THB	20.1			
	17-Deoxy-β-cortolone		22.1	10	463.3	373.5
	17-Deoxy-β-cortol		20.8	10	537.4	447.4
17-hydroxy steroids	Cortisone (E)		24.5	15	531.4	441.4
	Cortisol (F)	Epi-F	23.0	20	605.4	515.4
		F	23.7			
	5β-Dihydrocortisol (DHF)		21.7	15	607.5	517.3
	Tetrahydrocortisone		19.8	15	578.4	488.3
	Tetrahydrocortisol (THF)	Epi-THF	18.4	15	652.6	562.4
		5β-THF	18.8			
		5α-THF	19.1			
	Cortolones	β-cortolone	19.5	10	449.3	269.2
		α-cortolone	20.0			
	β-Cortol*		18.3	10	535.4	445.3

* Quantitation of α-cortol was not possible due to co-elution with 5α-THF.

Figure 2-2. Analysis of derivatised 17-deoxy β-cortol standard by GC-MS/MS in full scan (Q1) mode.

Panel A: total ion chromatogram demonstrating derivatised analyte elutes at 19.14 minutes. Panel B: Proposed structure of trimethylsilyl (TMS) derivative (MW 640) of 17-deoxy β-cortol (MW 352). Mass spectrum (panel C; t = 19.14 mins; *m/z* range 300-600) demonstrates peaks at 357.2 and 447.3, corresponding to loss of TMS groups (MW 90) from a fragment with *m/z* 537.4, itself consistent with elimination of TMS-OCH from the molecular ion.



2.3.8. Adipose biopsy real-time reverse transcription PCR (qRT-PCR)

RNA purification was performed according to manufacturer's instructions using an RNeasy® mini kit with QIAzol® lysis reagent (Qiagen, UK). RNA integrity was confirmed by presence of two clear bands when run on a denature get agarose gel, with the intensity of the larger 28S ribosomal RNA band approximately twice as strong that of 18S ribosomal RNA. cDNA (250 ng RNA/reaction) was synthesised using a high capacity cDNA kit (Invitrogen, Paisley, UK) according to manufacturer's instructions. Quantitative PCR (qPCR) was carried out using a Roche Lightcycler 480 (Roche Applied Science, Burgess Hill, UK). Primers (Invitrogen, Paisley, UK) were designed for use with intron-spanning probes (Roche Universal Probe Library (UPL®); Primer sequences and UPL® probe numbers in Table 2-2). Samples were initially denatured by heating (95 ºC, 5min) then underwent 50 cycles of PCR amplification comprising denaturation (95 ºC, 10 sec), annealing and elongation (60ºC, 30 sec). After the final cycle, samples were cooled (40 ºC, 30 sec). All samples were analysed in triplicate and amplification curves plotted (fluorescence vs axis cycle number). Triplicates were deemed acceptable if the standard deviation of crossing point (Cp) was less than 0.5 cycles. A standard curve for each gene was generated by serial dilution of cDNAs pooled from different samples, and deemed acceptable if reaction efficiency was between 1.7 and 2.1. Transcript abundance in samples (in triplicate) was quantified by interpolation from the standard curve and expressed as a factor of the housekeeping gene 18S ribosomal rRNA (18S rRNA).

Table 2-2. Primer sequence for qPCR and corresponding probe number from Roche Universal Probe Library (UPL)®.

Gene ID	Primer sequence 5' to 3'	Roche UPL® probe number
PER1 NM_002616.2	F: ctcttccacagctccctca	87
	R: ctttggatcggcagtggt	
LPL NM_000237.2	F: atgtggcccggtttatca	25
	R: ctgtatcccaagagatggacatt	
ADIPOQ NM_004797.3	F: ggtgagaagggtgagaaagga	85
	R: tttcaccgatgtctcccttag	
ATGL NM_020376.3	F: ctccaccaacatccacgag	89
	R: ccctgcttgcacatctctc	
PEPCK NM_002591.3	F: cgaaagctccccaagtacaa	20
	R: gctctctactcgtgccacatc	
18S rRNA	F: cttccacaggaggcctacac	46
	R: cgcaaaatatgctggaacttt	

Chapter 3: Corticosterone and Metabolic Syndrome

3.1. Introduction

In large-scale genome-wide association studies (GWAS) performed to identify genetic determinants of blood pressure, polymorphisms at a handful of key loci have been consistently associated with blood pressure in populations of various ancestries (Newton-Cheh et al, 2009; Kato et al, 2011). One of these is *CYP17A1*, which encodes the steroidogenic enzyme 17α-hydroxylase (Newton-Cheh et al, 2009; Ehret et al, 2011; Kato et al, 2011; Fedorowski et al, 2012; Hotta et al, 2012). In the adrenal gland, CYP17 is required for synthesis of the glucocorticoid hormone cortisol, whereas substrate that does not undergo 17-hydroxylation is converted to the mineralocorticoids 11-deoxycorticosterone (DOC) and aldosterone. Additionally, however, the 'mineralocorticoid' pathway also produces the glucocorticoid hormone corticosterone, which circulates at concentrations of 5-10 % of those of cortisol. The product of *CYP17A1* is therefore likely to be a key determinant of relative corticosterone vs cortisol production.

The mechanisms underlying associations of variability in *CYP17A1* with blood pressure have not been identified. Rare genetic variation, with near-absent 17α-hydroxylase activity, results in low-renin hypertension, associated with DOC and corticosterone excess (Yanase et al, 1991). It is therefore widely inferred that associations of more common variability in *CYP17A1* with increased blood pressure are mediated by reduced 17α-hydroxylase activity.

Raised blood pressure is a key component of the 'metabolic syndrome', the collection of risk factors for cardiovascular disease, which also includes obesity,

dyslipidaemia and insulin resistance. Intriguingly, risk alleles for hypertension are not associated with other features of metabolic syndrome, and in fact are associated with reduced risk of obesity (Hotta et al, 2012; Wen et al, 2014). Polymorphism in *CYP17A1* that predicts obesity has also been shown to associate with insulin resistance (Echiburu et al, 2008). Therefore, alleles that are expected to result in relatively impaired CYP17 activity and increased ratio of corticosterone: cortisol are associated with increased blood pressure but with reduced risk of obesity, and perhaps also insulin resistance.

This apparent paradox might be explained by relatively recent evidence, which points to a mechanism whereby increased production of corticosterone over cortisol may result in a favourable cardiometabolic profile. Metabolic syndrome is associated with activation of the HPA axis, (Pasquali et al, 2006), occurring through unknown mechanisms. HPA axis activation drives cortisol excess, which has clear adverse metabolic effects (Walker, 2007). Corticosterone, unlike cortisol, is not exported from the CNS by the transmembrane protein ABCB1 (Karssen et al, 2001). Therefore relative deficiency of corticosterone may result in impaired negative feedback suppression of the HPA axis and drive elevated plasma cortisol in metabolic syndrome.

The balance of corticosterone vs cortisol production may therefore be an important determinant of risk of metabolic syndrome, but the direction of association for blood pressure may differ to other components of the syndrome. In the studies presented in the current chapter, we aimed to determine the associations of plasma corticosterone vs cortisol with features of metabolic syndrome. We studied a large UK based

population cohort, the Orkney Complex Disease Study (ORCADES) group; a cohort of people with diabetes, the Edinburgh Type 2 Diabetes Study (ET2DS); and a smaller cohort of men and women who had undergone dynamic testing of the HPA axis, the East Hertfordshire (EHERTS) Study.

3.2. Methods

3.2.1. Subjects

All studies conformed to the Declaration of Helsinki and ethical approval and written informed consent were obtained.

3.2.1.1. Orkney Complex Disease Study (ORCADES)

The ORCADES study is a genetic epidemiological study based in the Scottish archipelago of Orkney, comprising 2039 subjects 18-100 years of age, with at least two Orcadian grandparents, recruited between 2005 and 2011 (McQuillan et al, 2008). Subjects attended a local or mobile venesection clinic between 0730h and 1100h, after fasting from 2200h the previous night. On a separate occasion subjects attended a cardiovascular measurement clinic where weight, height and blood pressure were recorded.

3.2.1.2. Edinburgh Type 2 Diabetes Study (ET2DS)

The Edinburgh Type 2 Diabetes Study (ET2DS) is a prospective cohort study comprising 1066 men and women with established type 2 diabetes, living in the Lothian region of Scotland. Recruitment and study design has been reported previously (Price et al, 2008). Briefly, participants age 60-75 years with a diagnosis of type 2 diabetes according to WHO criteria (Alberti et al, 1998) were contacted using data from computerised records. Following an overnight fast, subjects attended a local research clinic at 0800-0830h where they underwent venepuncture and physical examination.

3.2.1.3. East Hertfordshire Study (EHERTS)

All births in East Hertfordshire from 1911 onwards were notified by the attending midwife (Barker et al, 1989). As previously described, these records were used to recruit 309 women and 370 men, with no history of diabetes, for a standard 75 g oral glucose tolerance test (OGTT), performed at a local clinic at 0800-1020h following an overnight fast from 2100h (Hales et al, 1991). In a follow up study (Reynolds et al, 2001a), 312 of these individuals, born between 1920 and 1930, ingested 0.25 mg dexamethasone at 2200h, fasted overnight and attended a local clinic at 0830h the next morning. A baseline blood sample was obtained before 1.0 μg freshly diluted $ACTH_{1-24}$ (tetracosactrin, Synacthen, Alliance, Chippenham, UK) was administered intravenously, and repeat sampling was undertaken after 30 minutes. In both EHERTS and ORCADES, the homeostatic model assessment was used to quantify insulin resistance (HOMA-IR) (Turner et al, 1979).

3.2.2. Laboratory analyses

Serum cortisol was measured by radioimmunoassay with Guildhay antisera (Moore et al, 1985). After exclusion of individuals prescribed glucocorticoid therapy (ORCADES n = 21; ETD2S n = 163; EHERTS n = 33), there was sufficient sample for analysis of corticosterone in 2018 individuals from ORCADES; 903 in ET2DS; and 279 in EHERTS.

3.3. Statistical Analysis

All statistical analyses were undertaken using Minitab (version 16; State College PA). Continuous response variables were log transformed to obtain normal distributions. Student's t-test (paired where appropriate) was used for group comparisons of corticosterone/cortisol ratios and responses to ACTH.

Simple linear regression analysis of corticosterone or cortisol with a given 'response' (or dependent) variable was used to determine unadjusted p values for the regression coefficient. Analyses were then repeated with potentially confounding co-variables in multiple regression analyses. All analyses were adjusted for age and gender. Because the clearance of glucocorticoids is increased in obesity, BMI was included in all analyses as a predictor variable. Additionally, because timing of fasting samples varied in the ORCADES and EHERTS cohorts, venesection time (expressed as minutes after the first sample in the cohort) was included as a predictor variable in all models, as previously described (Reynolds et al, 2006), in order to adjust for diurnal variation in glucocorticoid production (and to adjust for variations in the interval between dexamethasone administration and time of sample in EHERTS).

Histograms and normal plots of residuals were examined to confirm the validity of the linear regression model. In order to maintain this, and to minimise confounding due to prescribed medication, those prescribed glucose-lowering medications were excluded from regression analyses involving glucose or insulin in the ORCADES and EHERTS cohorts. Because the majority of participants in ET2DS were prescribed these agents, their prescription was encoded as a binary variable and

entered into the regression equation. This approach was used in all cohorts to adjust for the effect of relevant medications on lipids and blood pressure.

In order to represent the magnitude of association of corticosterone and cortisol with a given response variable, the regression model was applied to fixed values of each glucocorticoid which represented the 1st and 3rd quartiles for the given cohort; this was preferred to calculating the change for each unit change in *Z* score, because of the non-normal distribution of cortisol and corticosterone values. The standard error of the regression coefficient was used to calculate the standard error of the modelled change in response variable. Results refer to adjusted analyses unless otherwise stated.

3.4. Results

Characteristics of the cohorts are summarised in Table 3-1.

Table 3-1. Characteristics of participants in epidemiological studies

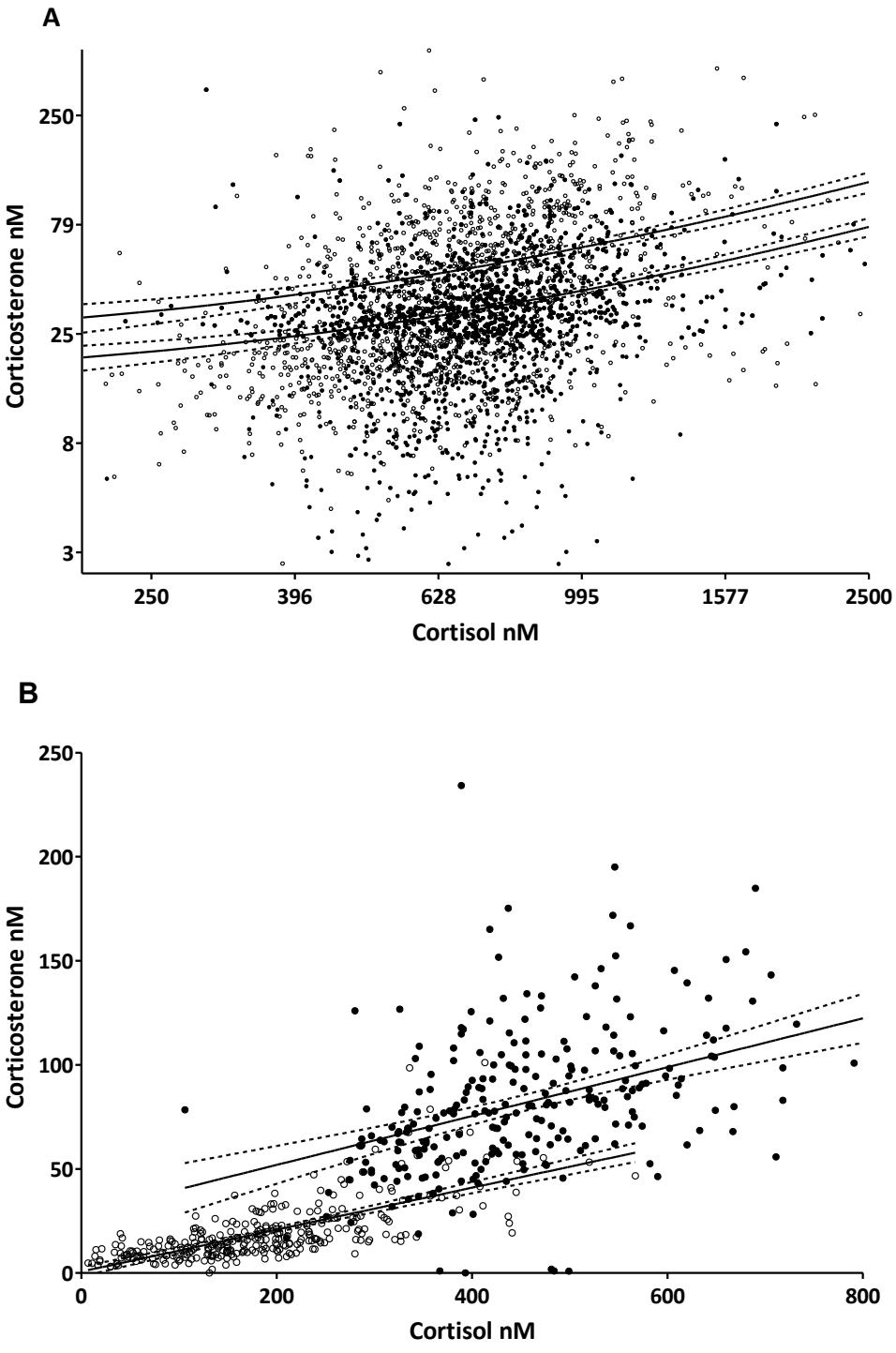
ORCADES = Orkney complex diseases study; ET2DS = Edinburgh type 2 diabetes study; EHERTS = East Hertfordshire study. Baseline glucocorticoid concentrations measured after dexamethasone suppression (0.25 mg) in EHERTS cohort. Glucose values at 30 and 120 mins refer to oral glucose tolerance test (75 g). Data presented as mean ± SEM. n/a = not available.

	Cohort		
	ORCADES	EHERTS	ET2DS
Gender (M/F)	794/1224 (39/61%)	189/90 (68/32%)	476/427 (53/47%)
Age (years)	53.3 ± 0.34 (16-91)	71.3 ± 0.18 (67-78)	67.9 ± 0.14 (60-68)
Body mass index (kg/m^2)	27.7 ± 0.11	27.1 ± 0.23	31.2 ± 0.19
Anti-hypertensive medication (n (%))	412 (20%)	105 (38%)	704 (79%)
Systolic BP (mmHg)	129.9 ± 0.43	159.3 ± 1.3	133.3 ± 0.55
Diastolic BP (mmHg)	75.5 ± 0.21	86.9 ± 0.69	69.2 ± 0.34
Corticosterone at baseline (nM)	50.7 ± 1.2	20.2 ± 1.0	22.3 ± 0.49
Corticosterone 30 mins after $ACTH_{1-24}$ (nM)	n/a	84.0 ± 2.5	n/a
Cortisol at baseline (nM)	724 ± 7.4	188 ± 6.1	732 ± 6.4
Cortisol 30 mins after $ACTH_{1-24}$ (nM)	n/a	460 ± 7.6	n/a
Lipid lowering therapy (n (%))	255 (13%)	71 (25%)	755 (84%)
Total cholesterol (mM)	5.39 ± 0.03	6.82 ± 0.07	4.28 ± 0.03
LDL cholesterol (mM)	3.37 ± 0.02	4.89 ± 0.08	n/a
HDL cholesterol (mM)	1.50 ± 0.01	1.33 ± 0.02	1.28 ± 0.01
Triglycerides (mM)	1.15 ± 0.02	1.54 ± 0.05	n/a
Medication for diabetes (n)	47 (2.3%)	10 (3.6%)	734 (81%)
Oral agents			660 (74%)
Insulin			156 (18%)
Fasting plasma glucose (mM)	5.33 ± 0.02	5.99 ± 0.08	7.57 ± 0.07
30 min plasma glucose (mM)	n/a	9.38 ± 0.14	n/a
120 min plasma glucose (mM)	n/a	7.13 ± 0.18	n/a
Fasting plasma insulin (mU/L)	6.44 ± 0.10	7.18 ± 0.35	n/a
HOMA-IR	1.59 ± 0.34	1.96 ± 0.11	n/a

Cortisol and corticosterone were positively correlated in the ORCADES and ET2DS cohorts (Figure 3-1A); and in both dexamethasone-suppressed and $ACTH_{1-24}$-stimulated samples in EHERTS (Figure 3-1B). After $ACTH_{1-24}$, the response of plasma corticosterone was greater than that of cortisol in EHERTS (6.2 ± 0.3 vs 3.9 ± 0.4 fold increase, respectively; $p < 0.001$).

Figure 3-1. Relationships between plasma cortisol and corticosterone.

See over for legend



A) Plasma cortisol and corticosterone in the Orkney complex diseases study group (ORCADES, open symbols) and the Edinburgh Type 2 Diabetes Study (ET2DS, closed symbols). Note log scale of axes. Lines indicate simple linear regression with 95% confidence bands. Coefficient of determination (r^2): ORCADES = 0.062, p<0.001; ET2DS = 0.070, p<0.001.

B) Cortisol and corticosterone in the East Hertfordshire Cohort. Sampling was undertaken following overnight dexamethasone (0.25 mg) suppression testing (pre synacthen, open symbols); and 30 minutes following intravenous injection of $ACTH_{1-24}$ (Synacthen 1 µg, closed symbols). Lines indicate simple linear regression with 95% confidence bands. r^2: pre-synacthen = 0.488 p<0.001; post synacthen = 0.168, p<0.001.

3.4.1. ORCADES

See Table 3-2 and Figure 3-2A. Both higher plasma cortisol and higher plasma corticosterone were associated with lower body mass index, higher triglycerides and higher fasting plasma glucose. Raised cortisol, but not corticosterone, was associated with raised systolic blood pressure. Neither cortisol nor corticosterone were significantly associated with total cholesterol or LDL cholesterol after adjustment for co-variables, although higher cortisol was more strikingly associated with higher triglyceride levels while higher corticosterone was associated with paradoxically higher HDL-cholesterol. Only plasma corticosterone was statistically significantly positively associated with fasting insulin and HOMA-IR, although similar trends were observed for plasma cortisol. Consistent with this, higher corticosterone: cortisol ratio was associated with higher fasting glucose, insulin and HOMA-IR.

3.4.2. ET2DS

See Table 3-2 and Figure 3-2B. In people with type 2 diabetes, higher plasma cortisol and plasma corticosterone were associated with lower BMI and higher

fasting plasma glucose, but were unrelated to systolic blood pressure. Corticosterone to cortisol ratio was not significantly associated with BMI, blood pressure or glucose.

3.4.3. EHERTS

See Table 3-2 and Figure 3-2C & D. Following low dose overnight dexamethasone suppression followed by low dose $ACTH_{1-24}$ administration, plasma cortisol and corticosterone levels changed as expected (Table 1). Plasma cortisol was not associated with any features of metabolic syndrome, except that $ACTH_{1-24}$-stimulated values were positively correlated with plasma glucose 30 min after an oral glucose load. However, plasma corticosterone showed altered associations with metabolic syndrome variables. After dexamethasone suppression, higher plasma corticosterone was associated with lower BMI but a paradoxically lower fasting insulin and HOMA-IR. After $ACTH_{1-24}$ stimulation, higher plasma corticosterone (and corticosterone/cortisol ratio) was associated with paradoxically lower glucose before and after an oral glucose load, and with lower HOMA-IR.

Table 3-2. Associations of plasma cortisol and corticosterone with features of the metabolic syndrome

ORCADES = Orkney complex diseases study; ET2DS = Edinburgh type 2 diabetes study; EHERTS = East Hertfordshire study. P values refer to regression co-efficient (β), unadjusted or adjusted in multiple regression analyses for co-variables (age, gender, BMI, time of sample, relevant medication). Glucose values at 30 min and 120 mins refer to oral glucose tolerance test (75 g). All continuous variables normalised by log transformation.

		Cortisol				Corticosterone				Corticosterone/cortisol ratio			
		Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
		β	p	β	p	β	p	β	p	β	p	β	p
ORCADES (n=2018)	BMI	-0.069	<0.001	-0.064	<0.001	-0.011	<0.001	-0.012	<0.001	-0.006	0.33	-0.009	0.11
	Systolic BP	-0.021	0.01	0.022	0.001	-0.002	0.41	0.002	0.25	0.003	0.48	-0.002	0.57
	Total cholesterol	-0.006	0.61	0.021	0.09	-0.004	0.15	-0.011	0.47	-0.010	0.17	-0.008	0.27
	LDL cholesterol	-0.042	0.02	0.006	0.74	-0.013	0.004	-0.008	0.08	-0.021	0.06	-0.018	0.09
	HDL cholesterol	0.063	<0.001	0.022	0.14	0.011	0.004	0.090	0.02	0.007	0.45	0.014	0.12
	Triglycerides	0.047	0.12	0.176	<0.001	0.007	0.35	0.025	<0.001	0.012	0.52	0.010	0.56
	Fasting glucose	-0.007	0.31	0.014	0.02	0.005	0.003	0.006	<0.001	0.015	<0.001	0.011	0.004
	Insulin	-0.066	0.03	0.030	0.27	0.004	0.58	0.018	0.01	0.038	0.03	0.038	0.02
	HOMA-IR	-0.073	0.03	0.045	0.13	0.009	0.27	0.025	0.001	0.053	0.01	0.048	0.01
ET2DS (n=903)	BMI	-0.064	0.003	-0.055	0.01	-0.018	0.05	-0.013	0.12	-0.010	0.30	-0.006	0.50
	Systolic BP	0.015	0.34	0.014	0.37	-0.003	0.66	-0.003	0.66	-0.005	0.40	-0.005	0.42
	Fasting glucose	0.179	<0.001	0.190	<0.001	0.027	0.03	0.030	0.02	0.001	0.95	0.003	0.84
EHERTS dexamethasone suppressed (n=279)	BMI	-0.018	0.13	-0.017	0.18	-0.036	0.003	-0.036	0.004	-0.023	0.14	-0.024	0.12
	Systolic BP	0.008	0.49	0.005	0.67	0.008	0.49	0.008	0.49	-0.002	0.89	0.000	1.00
	Fasting glucose	0.018	0.06	0.010	0.28	0.003	0.74	-0.001	0.89	-0.019	0.12	-0.012	0.30
	30 min glucose	0.033	0.05	0.027	0.12	0.015	0.39	0.015	0.38	-0.027	0.23	-0.014	0.51
	120 min glucose	-0.049	0.04	-0.034	0.17	-0.050	0.04	-0.032	0.20	0.016	0.61	0.012	0.69
	Insulin	-0.138	0.01	-0.096	0.06	-0.192	<0.001	-0.131	0.01	-0.046	0.80	-0.040	0.52
	HOMA-IR	-0.119	0.03	-0.085	0.11	-0.189	0.001	-0.133	0.01	-0.105	0.59	-0.053	0.42
EHERTS after $ACTH_{1-24}$ (n=279)	BMI	0.012	0.71	-0.020	0.61	-0.015	0.19	-0.018	0.12	-0.019	0.11	-0.017	0.15
	Systolic BP	0.014	0.65	0.048	0.19	-0.007	0.58	-0.004	0.77	-0.014	0.32	-0.011	0.41
	Fasting glucose	-0.031	0.19	0.037	0.20	-0.033	0.003	-0.027	0.01	-0.021	0.06	-0.026	0.02
	30 min glucose	0.019	0.66	0.146	0.01	-0.023	0.25	-0.010	0.63	-0.021	0.32	-0.019	0.35
	120 min glucose	0.092	0.15	0.010	0.90	-0.054	0.06	-0.056	0.05	-0.072	0.02	-0.059	0.05
	Insulin	0.136	0.32	0.036	0.82	-0.120	0.04	-0.099	0.07	-0.046	0.02	-0.096	0.09
	HOMA-IR	0.103	0.48	0.072	0.66	-0.153	0.01	-0.126	0.03	-0.485	0.02	-0.123	0.04

Figure 3-2. Associations of plasma cortisol and corticosterone with features of metabolic syndrome. See over for legend

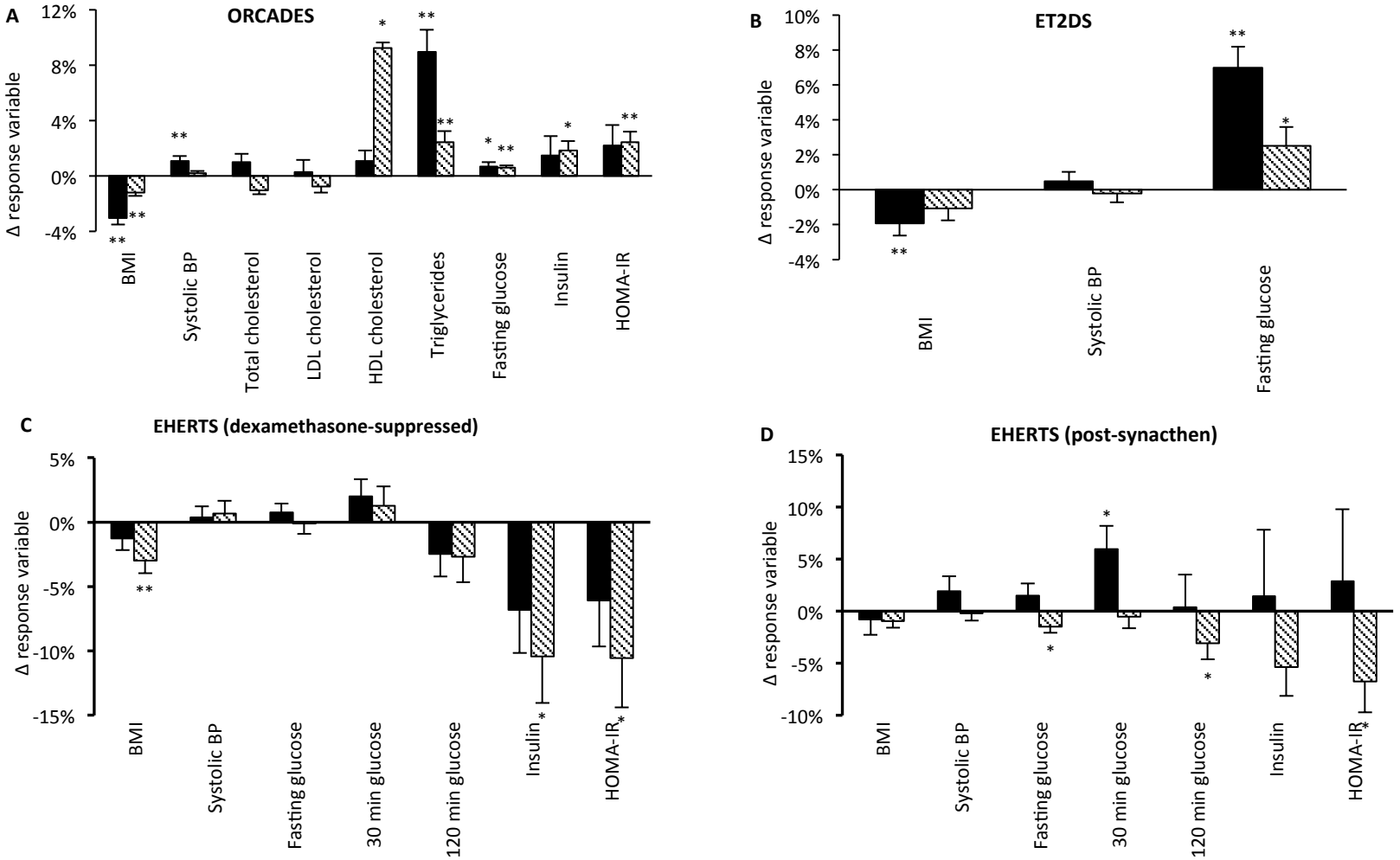


Figure 3-2. Associations of plasma cortisol and corticosterone with features of metabolic syndrome

Data are from A) Orkney complex diseases study (ORCADES), B) Edinburgh Type 2 Diabetes Study (ET2DS), C) East Hertfordshire study (EHERTS) after overnight dexamethasone (0.25 mg) suppression, and D) EHERTS 30 mins after $ACTH_{1-24}$ (1 μg). Results are for the % change in each response variable comparing the lower and upper tertile cut-off values for cortisol (filled columns) or corticosterone (hatched columns), calculated from the adjusted regression coefficients shown in Table 3-2. For corticosterone, tertile cut-offs were 23 and 61 nM in ORCADES, 13 and 29 nM in ET2DS, 10 and 24 nM in EHERTS after dexamethasone, and 60 and 104 nM after $ACTH_{1-24}$. For cortisol, tertile cut-offs were 521 and 847 nM in ORCADES, 591 and 842 nM in ET2DS, 117 and 245 nM in EHERTS after dexamethasone, and 366 and 544 nM after $ACTH_{1-24}$. Error bars were calculated from upper or lower range of standard error of the regression coefficient. *$p < 0.05$, **$p < 0.005$

3.5. Discussion

This is the largest study investigating the associations between endogenous glucocorticoids and features of metabolic syndrome. By analysing fasting morning plasma samples from 2 cohorts comprising 2921 participants, we confirm previous observations (Filipovsky et al, 1996; Phillips et al, 1998b; Reynolds et al, 2001b; Reynolds et al, 2003; Ward et al, 2003; Reynolds et al, 2010; Krishnaveni et al, 2013) that elevated morning plasma cortisol is associated with higher blood pressure and blood glucose, but with lower body mass index, both in subjects with and without type 2 diabetes. With such large numbers of participants, the chances of statistical errors are much reduced compared with smaller previous studies of plasma cortisol.

This is the first study to investigate whether similar associations exist for plasma corticosterone. Population genetic data raise the hypothesis that reduced adrenal CYP17 activity, expected to increase the ratio of plasma corticosterone: cortisol, contributes to raised blood pressure. Our data demonstrate no associations between plasma corticosterone or the ratio of corticosterone: cortisol and blood pressure. Although we adjusted for the use of antihypertensive medication, their use may still limit the ability to test associations with blood pressure, especially in the ET2DS and EHERTS cohorts. Perhaps also of relevance to blood pressure, we also hypothesised that relative deficiency of corticosterone would aggravate cortisol excess and be associated with other features of metabolic syndrome. If such a mechanism existed, positive associations of corticosterone with blood pressure might be attenuated, or indeed obscured, by effects of corticosterone on the HPA axis, which would act to

limit cortisol excess and adverse effects on vascular smooth muscle (Sapolsky et al, 2000).

Seemingly against this hypothesis, our data show that in basal samples, associations of plasma corticosterone with fasting insulin and glucose are similar to those of cortisol, and in fact the ratio of corticosterone: cortisol indicates associations are greater for corticosterone than cortisol. The stronger positive associations of corticosterone than cortisol with fasting insulin and glucose in non-diabetic subjects is intriguing. We are not aware of evidence that corticosterone could have a greater action than cortisol on insulin resistance or glucose metabolism, but this now deserves more careful investigation. Corticosterone and cortisol both bind corticosteroid-binding globulin, so changes in binding capacity in association with insulin resistance (Fernandez-Real et al, 2000; Fernandez-Real et al, 2002) are unlikely to explain stronger associations with corticosterone. The observation might be explained by 'reverse causality', whereby insulin resistance mediates a change in adrenal steroidogenesis in favour of corticosterone. It has been hypothesised that enhanced 17α-hydroxylase activity by insulin *in vitro* (Munir et al, 2004) explains adrenal hyperandrogenism in polycystic ovarian syndrome (Baptiste et al, 2010). This hypothesis assumes that the enzyme remains insulin-sensitive in an otherwise insulin resistant state; whereas loss of insulin-driven upregulation might in fact shift adrenal steroidogenesis towards corticosterone. Alternatively, the combined elevation of cortisol and corticosterone with insulin resistance could reflect a central activation of the HPA axis, consistent with dysregulation of central control exemplified by altered responses to habituation and stress (Reynolds et al, 2001b).

In contrast to associations of basal samples, data from EHERTS indicate insulin sensitive individuals show a tendency to produce corticosterone over cortisol following $ACTH_{1-24}$ stimulation, as indicated by inverse associations of the corticosterone: cortisol ratio with fasting glucose, glucose at 30 minutes in the OGTT, and HOMA-IR. Although these observation were made in a different cohort, with smaller numbers than the other cohorts, the properties of these samples means they provide a unique opportunity to test the hypothesis that variability in CYP17 results in dysregulation of adrenal steroidogenesis, whereby a biosynthetic bias towards corticosterone over cortisol predicts a favourable metabolic profile. Basal glucocorticoids are prone to confounding by HPA axis tone: relative impairment in adrenocortical secretion of corticosterone may result in compensatory activation of the HPA axis to normalise corticosterone and elevate cortisol, potentially obscuring associations of plasma corticosterone with metabolic syndrome. Changes in clearance that differentially affect corticosterone and cortisol may have similar effects. Dexamethasone suppression followed by $ACTH_{1-24}$ stimulation effectively 'normalises' HPA axis tone across the cohort, and these samples therefore more accurately reflect the innate adrenal biosynthetic tendency towards corticosterone vs cortisol production. If, as a result of the activity of ABCB1 excluding cortisol from the brain (Karssen et al, 2001) corticosterone makes a disproportionate contribution to negative feedback suppression of the HPA axis in humans, these observations could provide a key insight into the activation of the HPA axis and elevated plasma cortisol in metabolic syndrome.

In dexamethasone-suppressed, pre-$ACTH_{1-24}$ samples from EHERTS, we found no significant associations between cortisol and features of metabolic syndrome. This finding is in keeping with previous studies, which generally show dexamethasone-suppressed cortisol fails to demonstrate associations with metabolic syndrome variables (Abraham et al, 2013). We did, however, demonstrate insulin resistance is accompanied by relatively deficiency of corticosterone, similar to samples following $ACTH_{1-24}$ stimulation.

Interestingly, in the diabetic cohort, fasting plasma corticosterone was low, whereas cortisol was similar to the values in the ORCADES cohort (mean corticosterone/cortisol ratio 0.03 in ET2DS vs 0.075 in ORCADES, $p < 0.0001$). It is unlikely that this phenomenon is due to the older age of the ET2DS cohort, since corticosterone/cortisol ratio was not associated with age ($r^2 = 0.01$, $p = 0.27$). Within the diabetic group, corticosterone and cortisol were both associated with BMI and fasting plasma glucose, but corticosterone was not more strongly correlated with blood glucose, as it was amongst non-diabetics in ORCADES. These findings cannot easily be explained by reverse causality (Baptiste et al, 2010). Confounding by HPA axis activation and/or alterations in clearance may explain the lesser positive associations of corticosterone with hyperglycaemia in this patient group.

Our data show corticosterone and cortisol are both reduced in obesity, and the ratio of corticosterone: cortisol is not associated with BMI. Both glucocorticoids are subject to similar routes of metabolism. It is likely that reduced plasma corticosterone in obesity is explained by the same factors which underlie increased clearance of cortisol in obesity, namely increased metabolism, eg by A-ring

reductases (Strain et al, 1980; Andrew et al, 1998; Lottenberg et al, 1998). Additionally, we found no clear association between the metabolic syndrome pattern of blood cholesterol and morning plasma glucocorticoid levels. The dyslipidaemia that accompanies metabolic syndrome is well characterised, and comprises raised total and LDL cholesterol, low HDL cholesterol and raised triglycerides, but previous studies have found inconsistent relationships between endogenous glucocorticoids and lipids. The positive association between corticosterone and HDL cholesterol is difficult to explain, since previous studies have shown high (Taskinen et al, 1983), low (Colao et al, 1999) or unchanged (Friedman et al, 1996) HDL cholesterol in Cushing's syndrome. Elevated triglycerides, which are more closely responsive to insulin signalling, were consistently associated with both cortisol and corticosterone.

An important limitation of these studies is the cross-sectional design, which means it is not possible to infer causation. Moreover, the dynamic HPA axis tests were conducted in a different cohort from the measurement of fasting plasma glucocorticoids. However, we have been able to draw upon comprehensive datasets to account for a number of potentially confounding variables. Heterogeneity between the cohorts means it is difficult to draw comparisons between the study groups, and ideally the findings require replication in similar representative cohorts.

In conclusion, we have demonstrated that elevated plasma corticosterone accompanies insulin resistance and elevated cortisol in metabolic syndrome, consistent with chronic up-regulation of the HPA axis. However, discrepancies between associations with cortisol and corticosterone may reflect differences in

adrenal steroidogenesis, particularly by 17α-hydroxylase, and may be a consequence of dysregulated insulin signaling. These findings throw the spotlight on corticosterone and suggest that further dissection of its biology in humans may be fruitful in understanding the basis for altered glucocorticoid signalling in metabolic syndrome.

Chapter 4: *In vitro* metabolism of corticosterone

4.1. Introduction and Aims

Clearance of glucocorticoids occurs in humans by two principal pathways: reversible oxidation at position C-11, catalysed by the 11β-HSDs (section 1.1.4); and step-wise reduction at positions 5, 3 and 20 (section 1.1.5), catalysed by hepatic A-ring reductases and HSDs. The importance of clearance by both pathways in determining glucocorticoid action is illustrated by the effects of therapeutic manipulation of the 11β-HSDs (Hughes et al, 2008); and by the recent finding that altered hepatic glucocorticoid clearance is a key component of the stress response (Boonen et al, 2013). The metabolism of cortisol by these pathways has been extensively studied in humans, both *in vitro* and *in vivo*; but in contrast little is known regarding the corresponding metabolic pathways for corticosterone (Figure 4-1), although it is thought to be similar in profile to cortisol.

In subsequent chapters, we aim to study the production and clearance of corticosterone *in vivo*, by analysis of urinary glucocorticoids and stable isotope tracer studies. For the latter, a deuterium labelled corticosterone tracer (D8-corticosterone; Figure 4-2) is commercially available (2,2,4,6,6,17α,21,21-[^{2}H]$_{8}$-corticosterone). Because this tracer has not previously been validated in humans, we undertook studies to test whether the compound exhibits an 'isotope effect', whereby the tracer differs in metabolism to the endogenous compound. Deuterium isotope effects are described as primary when the reaction involves cleavage of the bond to the isotopically labelled atom; and secondary when the D-H bond remains intact (Wiberg, 1955). *In vitro* studies presented in the current chapter aim to determine

the kinetic properties of the principal enzymes involved in the clearance of corticosterone, in order to complement studies of its *in vivo* physiology; and to validate the deuterated tracer intended for use in these studies.

Figure 4-1. Proposed metabolic pathways for the clearance of corticosterone in humans

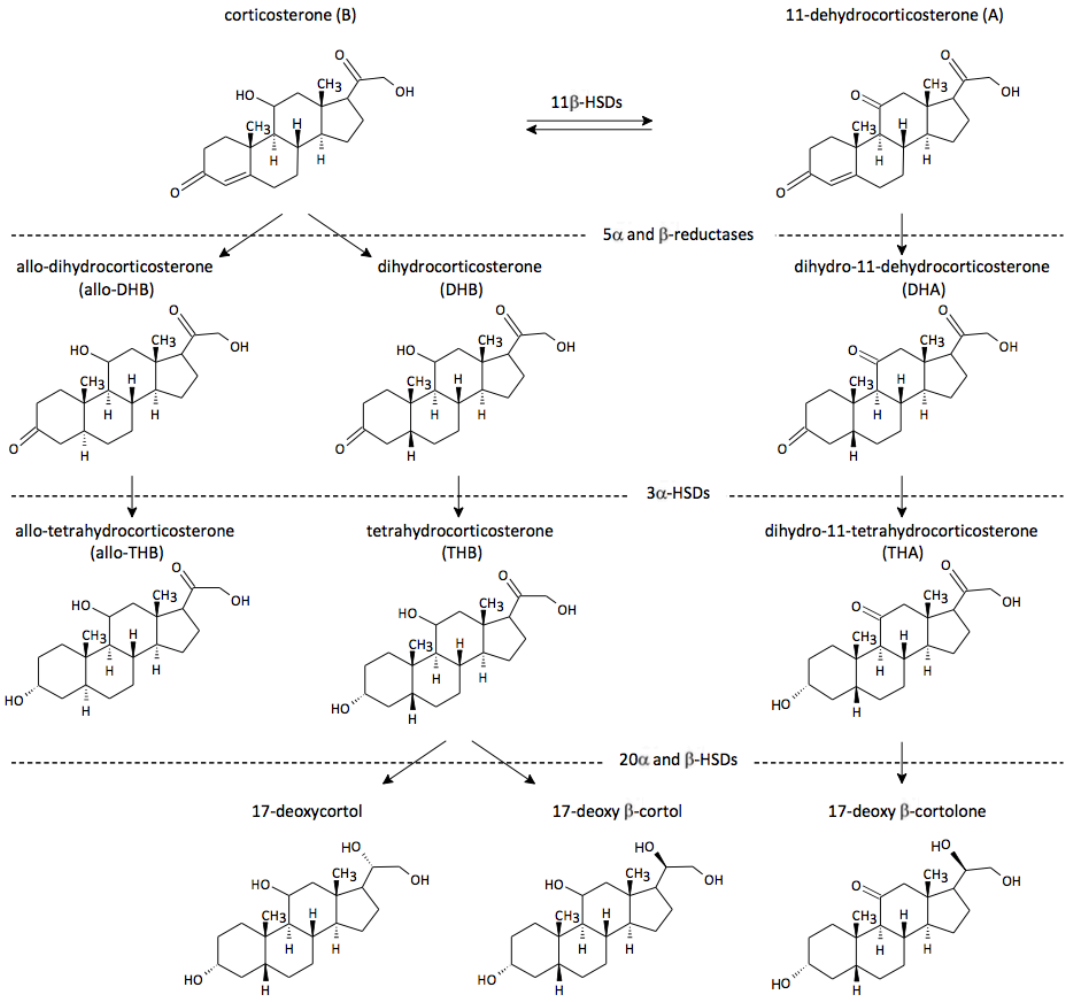
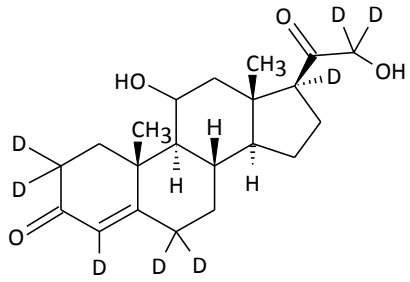


Figure 4-2. 2, 2, 4, 6, 6, 17α, 21, 21-[^{2}H]$_8$-corticosterone (D8-corticosterone)



4.1.1. Aims

- To validate the stable isotope D8-corticosterone as a tracer for corticosterone by testing for isotope effects in their metabolism by the key clearance enzymes 5β-reductase and 11β-HSD2
- To characterise the *in vitro* metabolism of corticosterone by 5β-reductase and 11β-HSD2; and compare it to that of cortisol

4.2. Methods

4.2.1. Metabolism of glucocorticoids by 5β-reductase

As 5β-reduced glucocorticoids are metabolised quickly by 3α-hydroxysteroid dehydrogenases (HSDs) *in vitro* (Iyer et al, 1990), it was not possible to assess metabolism of corticosterone by 5β-reductase and 3α-HSD individually. Therefore human hepatic cytosol was used to assess the combined action of these two enzymes resulting in formation of 3α, 5β-tetrahydrocorticosterone or its D8-labelled isotopologue. The assay was adapted from one reported to quantify 5β-reductase activity in rat (Okuda et al, 1984) and human liver (Iyer et al, 1990). Pilot experiments were performed in rodent hepatic homogenates to determine optimal protein concentration, unlabelled substrate concentration, incubation time and chromatographic conditions.

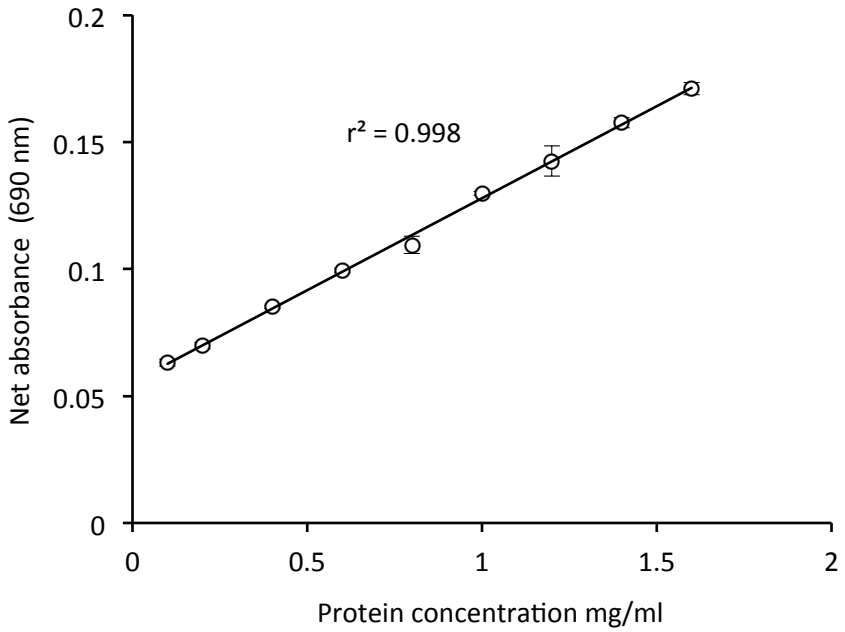
4.2.1.1. Determination of hepatic cytosol protein content

A colorimetric Bio-Rad DC® protein assay kit (Bio-Rad, Hemel Hempstead, UK) was used to determine protein concentration in hepatic cytosol. A stock protein solution (2 mg/ml BSA, fraction V, in water) was used to prepare standard solutions (0.1 - 1.4 mg/ml) in triplicate in distilled water. Protein standards (5 µl) were added to a 96-well plate in triplicate, with a solution of sodium dodecyl sulfate (SDS; 1 %) used as a blank. Hepatic cytosol samples of unknown protein concentration were also added in triplicate. Bio-Rad DC protein assay Reagents A (3 ml) and S (60 µl) were mixed to form working Reagent A. Working Reagent A (25 µl) then dye

Reagent B (200 µl) was added into each of the wells. The plate was allowed to stand (room temperature, 15 minutes) before determining absorbance of samples at λ 690 nm using an ultra-violet spectrophotometer. A standard curve was plotted with absorbance on the *y* axis vs concentration on the *x* axis, a line of best fit drawn using simple linear regression (Figure 4-3), and the regression equation was used to determine the concentration of protein in each sample. Data were deemed acceptable if the r^2 was >0.99 and coefficient of variation between triplicates was <10%.

Figure 4-3. Bio-Rad DC® protein assay: representative standard curve.

mean ± SEM (n = 3)



4.2.1.2. Metabolism of steroids by 5β-reductase/3α-HSD in human hepatic cytosol

5β-Reductase activity was assessed by incubating substrate (20 µl) with cytosol (180 µl) and co-factor mix (50 µl). Required volumes of stock substrate (in methanol) were dried under oxygen-free nitrogen (OFN; 60 °C), reconstituted in ethanol (50

µl), and vortexed thoroughly to ensure they were fully dissolved before adding potassium phosphate buffer (450 µl). Hepatic cytosol and co-factor mix were prepared in potassium phosphate buffer. Hepatic cytosol was required in a solution with a final concentration of 100 - 200 µg protein per assay volume (250 µl), and was therefore prepared initially at 0.56 - 1.11 µg protein/µl. Co-factor mix was prepared fresh for each assay and stored (4 °C) for no longer than 2 hours and protected from the light. G6P-DH was required at 2 units/ml in the final assay volume, and was therefore prepared initially at 10 units/ml. This was used to dissolve G6P (final concentration 5 mM; prepared initially at 25 mM); which was used in turn to dissolve NADPH (final concentration 2 mM; prepared initially at 10 mM). The co-factor mix was then incubated in a water bath (37 °C, 10 minutes) to establish reducing conditions.

The assay was started by mixing diluted cytosol (or assay buffer in 'no-protein' controls), co-factor mix (or assay buffer in 'no co-factor' controls), and substrate mix. Samples were covered and incubated in a shaking water bath (37 °C; 40 strokes/min). Time courses were performed to determine the optimum incubation period.

The reaction was terminated by adding 10 volumes (2.5 ml) ethyl acetate. The organic (top) layer was removed, reduced to dryness under OFN (60 °C), re-constituted in mobile phase (water: acetonitrile: methanol, 60:15:25) and transferred to high performance liquid chromatography (HPLC) vials (VWR International, Radnor, PA). Samples were stored (-20 °C) for analysis by HPLC with radio-detection (section 4.2.1.3). When the assay mix contained 20 nM

[$^3H_4$]-substrate, 30 µl was injected from samples prepared in 200 µl mobile phase. Using 5 nM [$^3H]_4$-substrate, 200 µl was injected from samples prepared in 250 µl mobile phase.

4.2.1.3. Analysis of glucocorticoids and metabolites by high performance liquid chromatography (HPLC) with radio-detection/UV detection

Analysis of tritiated steroids was achieved using a 626 HPLC pump (Waters, Milford, MA), ASI 100 automated sample injector (Dionex, Leeds, UK) and a β-scintillation counter (Berthold LB509 detector (Harpenden, UK). When simultaneous analysis of non-tritiated steroids was required, a 170U UV detector (Dionex, Leeds, UK) was employed. Substrate and product eluted at 35 °C using a 46 x 150 mm Sunfire C18 5 µM column (Waters, Milford, MA) under conditions described in Table 4-1. To achieve optimal mixing and counting efficiency the scintillant (ProFlow G; Biotechnologies Ltd, Epsom, UK) flow rate was 2.0 ml/ min.

Where possible, the product was identified by comparison of its retention time with that of tritiated standard. Radio-labelled standards were not available to confirm the identity of products of metabolism by hepatic cytosol; instead this was undertaken by analysis of unlabelled 3α, 5β-tetrahydro-steroid standard using UV detection (λ = 200 nm).

Table 4-1. Chromatographic conditions for the analysis of corticosterone (B), D8-corticosterone (D8-B), cortisol (F) and metabolites by HPLC.

		5β-reductase/3α-HSD assay		11β-HSD2 assay	
Substrate		B/D8-B	F	B/D8-B	F
Mobile phase composition (water: acetonitrile: methanol)		60:15:25	60:15:25	60:15:25	60:0:40
Mobile phase flow rate (ml/min)		1.5	1.5	1.5	1
Mobile phase volume (µl)		200 - 250	200 -250	250	250
Injection volume (µl)		30 - 200	30 - 200	60	60
Retention time (mins)	Substrate	21.8	12.2	21.4	34.7
	Product	32.6	22.5	14.7	27.8

4.2.1.4. Data analysis

Following chromatography, the area under each peak was integrated (Chromeleon version 6.8, Dionex, Leeds, UK) and the percentage conversion of tritiated substrate to tritiated product quantified and converted into substrate and product concentrations. Peak areas were integrated when the response had a signal to noise ratio (SNR) >3. Data points were accepted if coefficient of variation between replicates was <10 %. Kinetic parameters were derived using least squares curve fitting (GraphPad Prism, version 5.01, GraphPad Software Inc, La Jolla CA). The extra sum-of-squares F test was used to compare best fit parameters between substrates.

4.2.2. Metabolism of glucocorticoids by 11β-HSD2

Human embryonic kidney cells stably transfected with human *HSD11B2* (HEK 293 11BHSD2) were obtained from Dr Scott Webster and Mrs Margaret Binnie, University of Edinburgh (Webster et al, 2007). Practice assays were performed to

determine the optimum range of labelled and unlabelled substrate concentrations, assay volume, cell density and incubation period.

4.2.2.1. Cell culture conditions

Cells stored in liquid nitrogen were thawed at room temperature before adding Dulbecco's Modified Eagle's Medium (DMEM; 50 ml) and centrifuging (1000 x g, 5 mins, room temperature). Medium was removed and the cell pellet re-suspended in 30 ml fresh medium.

Cells were maintained in uncoated flasks (175 ml) containing DMEM (15 ml). Cells were passaged when confluent (approximately twice weekly). DMEM was aspirated from the flask and cells were washed with phosphate-buffered saline (PBS; 5-10 ml) to remove divalent cations and serum. The cells were enzymatically displaced by adding trypsin / EDTA (3 ml) and cells were incubated (37 °C, 3-5) minutes. Cells were dislodged and observed under the microscope. Trypsin was neutralised with an equal volume of medium. Cells were then centrifuged (1000 × g, 5 mins, room temperature) to remove any debris. Medium was removed and the cell pellet was re-suspended in fresh medium. The required volume of suspension was transferred into each flask and then topped-up to 15 ml of medium in total.

4.2.2.2. Assay

Cells were suspended in fresh medium and mixed well, before placing 200 µl onto a haemocytometer and covering with a cover slip. Cell count was determined from the average count of 4 × 100 nl volumes. Cells were seeded onto poly-D-lysine hydrobromide-coated 12 well plates (BD Biosciences, Oxford, UK) at a density of 5

$\times 10^5$ cells/well (using 2 ml DMEM/well) and incubated (20-24 hours, 37 °C). Unstripped medium was aspirated from the wells and cells were washed with PBS. Medium was replaced with DMEM containing stripped FCS (10% *v/v*; 2 ml) and cells were incubated (20-24 hours, 37 °C).

DMEM was aspirated from the wells and medium was replaced with 2 ml fresh serum-free DMEM, containing $[^3H]_4$-corticosterone (5 nM) and unlabelled substrate (2.5 - 45 nM) in duplicate). Controls were included which did not contain cells or substrate. Time courses were performed to determine the optimal incubation time (60-300 minutes; 37 °C) for each substrate. At the end of the incubation period, medium was aspirated, collected into 12 x 75 mm glass culture tubes and stored (-20 °C) before extraction of glucocorticoids.

4.2.2.3. Steroid Extraction from Cell Culture Medium

Glucocorticoids were extracted from cell culture medium using a Sep-Pak C18 column (360 mg; 55-105 µm, Waters, Milford, MA). Columns were conditioned under atmospheric pressure with methanol (5 ml) then water (5 ml). Cell culture medium was applied and the column was washed again with water (5 ml). Steroids were eluted using methanol (2 ml) and the eluate collected in 12 × 75 mm glass culture tubes. The eluate was reduced to dryness under 60 °C OFN and samples were reconstituted in water (200 µl). Steroids were extracted using ethyl acetate (2 ml). The organic layer was then reduced to dryness under 60 °C OFN; dissolved in mobile phase, transferred to HPLC vials, and stored (-20 °C) for analysis by HPLC (section 4.2.1.3 and Table 4-1).

4.3. Results

4.3.1. Metabolism of glucocorticoids by 5β-reductase/3α-HSD

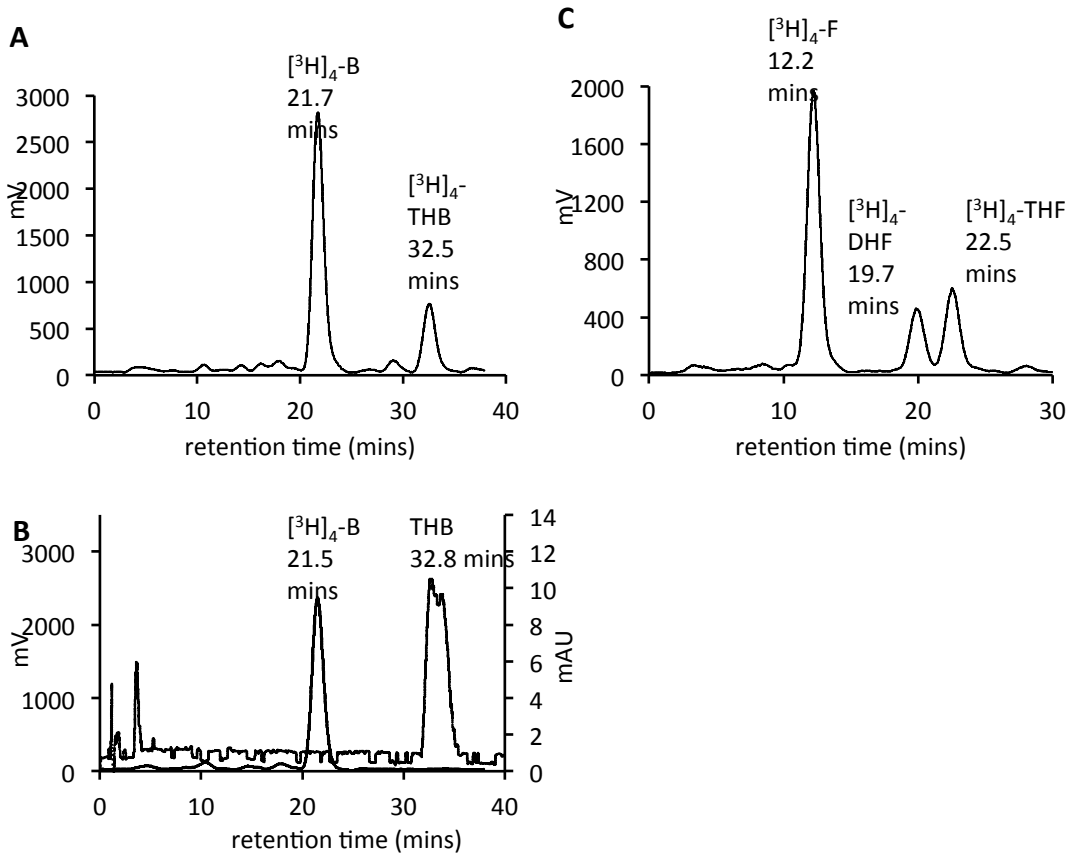
Representative radio-chromatograms demonstrating the metabolism of [^{3}H]-labelled corticosterone and cortisol by human hepatic cytosol are shown in Figure 4-4. [^{3}H]$_4$-products were not formed in control samples without cytosol or co-factor.

Unlabelled 3α, 5β-tetrahydrocortisol could not be detected using UV detection. Attempts were therefore made to analyse the compound by mass spectrometry. A Micromass ZMD single quadrupole mass spectrometer (Waters, Milford, MA) was used to determine the mass/charge (*m/z*) ratios of ions formed from the electrospray ionisation of infused standards (10 μg/ml in water: methanol: formic acid 50: 50: 0.1 %; 30 μl/min). Expected quasimolecular ions $[M + H]^+$ and $M + Na]^+$ were observed for cortisol but not reduced metabolites. Final confirmation of product was achieved by derivatisation and analysis by GC-MS/MS. Unlabelled substrate was incubated with hepatic cytosol under experimental conditions described (section 4.2.1.2) using 10 μM substrate. Substrate and product were then isolated by HPLC followed by collection of the fractions whose retention time matched those of the corresponding [^{3}H]$_4$ compounds. Eluates were reduced to dryness then processed and analysed as described in section 2.3.7.1. This confirmed the identity of the peaks at 22.5 and 32.5 minutes as tetrahydrocortisol and tetrahydrocorticosterone respectively (Figure 4-4). The rate of formation of additional products, eluting approximately 0.8 minutes before tetrahydro-reduced compounds, was not linear within the period studied. Analysis by GC-MS/MS confirmed this peak corresponded to

5β-dihydrocortisol (where the substrate was cortisol), but in the case of corticosterone, the identity of the peak, which was less abundant, could not be confirmed.

Figure 4-4. Metabolism of corticosterone and cortisol by 5β-reductase/3α-HSD: representative chromatograms

Panel A: Representative radio-chromatogram following incubation of corticosterone (5 µM) with $[^3H]_4$-corticosterone (2 nM) and human hepatic cytosol (100 µg protein) for 5 hours. Panel B: $[^3H]_4$-corticosterone standard (analysed by radio-detection; left axis) co-infused with unlabelled 3α, 5β-tetrahydrocorticosterone (detected by absorbance at 200 nm; right axis). Panel C: Representative radio-chromatogram following incubation of cortisol (10 µM) with $[^3H]_4$-cortisol (10 nM) and human hepatic cytosol (200 µg protein) for 4 hours.

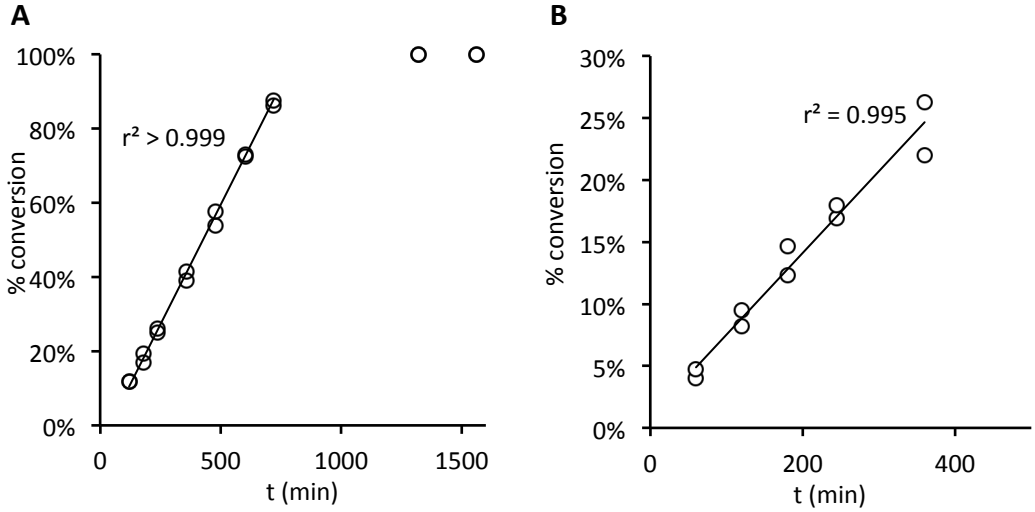


Rate of product formation over time was linear over the period 120-720 minutes for incubations with $[^3H]_4$-corticosterone and 60-360 minutes for $[^3H]_4$-cortisol (Figure

4-5). Time points for subsequent assays were selected in order to achieve 10-40 % conversion of substrate.

Figure 4-5. Metabolism of corticosterone and cortisol by human 5β-reductase/3α-HSD: time course

Glucocorticoid incubated in duplicate with human hepatic cytosol (200 µg protein) using corticosterone (10 µM) and $[^3H]_4$-corticosterone (20 nM) as substrate (panel A); or $[^3H]_4$-cortisol (10 nM) alone (panel B).



4.3.1.1. Tracer validation: corticosterone vs D8-corticosterone

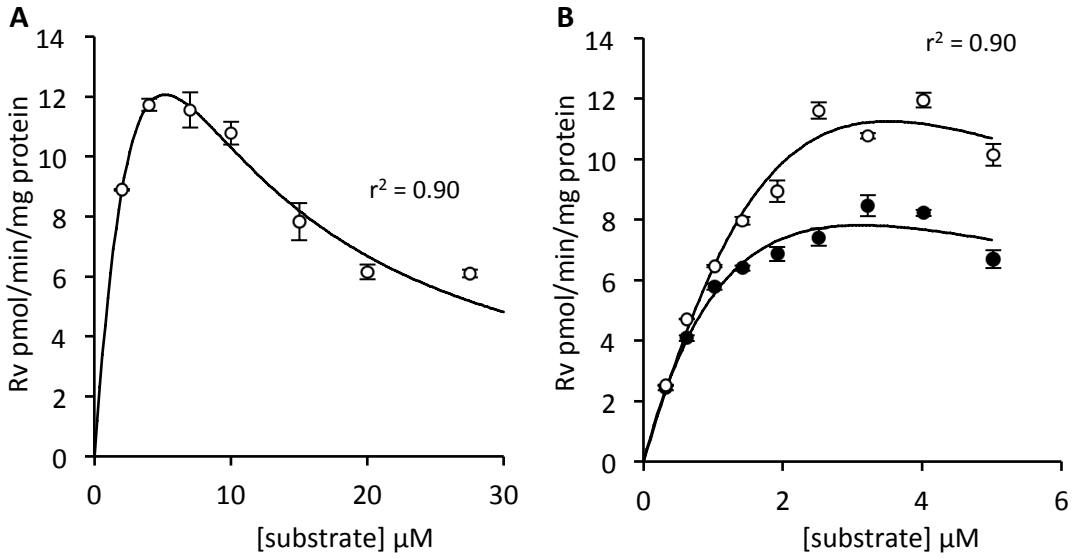
Both corticosterone and D8-corticosterone were substrates for human hepatic 5β-reductase/3α-HSD. At supraphysiological substrate concentrations (> 6 µM), rate of metabolism declined with increasing concentration of both isotopologues (Figure 4-6; panel A). Enzyme kinetics were best modelled according to the Michaelis-Menten equation adjusted for substrate inhibition (Copeland, 2000):

$$\boldsymbol{v} = \frac{V_{max}[S]}{K_m + [S] + \frac{[S]^2}{K_i}}$$

At higher substrate concentrations, rate of metabolism of corticosterone (Figure 4-6; panels A and B) exceeded that of D8-corticosterone ($p < 0.001$), indicating a deuterium primary isotope effect. The magnitude of this effect, expressed as the ratio of peak modelled reaction velocities k_H/k_D was 1.44.

Figure 4-6. Tracer validation: metabolism of corticosterone and D8-corticosterone by 5β-reductase/3α-HSD - Michaelis-Menten kinetics modified for substrate inhibition

Panel A: corticosterone (2-27.5 µM) and $[^3H]_4$-corticosterone (20 nM) incubated in triplicate with human hepatic cytosol (200 µg protein). Panel B: corticosterone (○) or D8-corticosterone (●) 0.3-5 µM incubated for 120-300 minutes with human hepatic cytosol (100 µg protein) and $[^3H]_4$-corticosterone (20 nM). Rv = reaction velocity. N = 3, mean ± SEM.



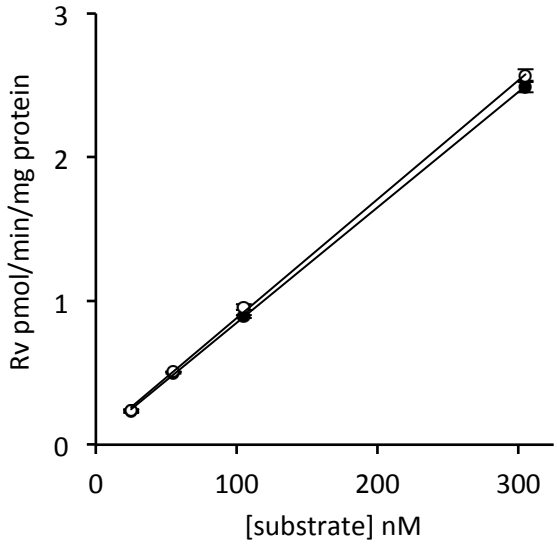
[Substrate] µM	Substrate	V_{max} (pmol/min/mg protein)	K_m (µM)	K_i (µM)	r^2
2 – 27.5	Corticosterone	50 ± 32	8.2 ± 5.0	3.3 ± 1.8	0.90
0.3 - 5	Corticosterone*	72 ± 60	9.5 ± 8.9	1.3 ± 1.4	0.96
	D8-corticosterone*	18 ± 5	2.2 ± 0.8	4.5 ± 2.3	0.93

* $p<0.001$ (extra sum-of-squares F test; null hypothesis = single curve for both substrates)

Repeat experiments undertaken within the physiological range revealed reaction velocity increased linearly ($r^2 > 0.99$) with increasing substrate concentration (Figure 4-6), and the gradient of the line of best fit for reaction velocity vs substrate concentration was not significantly different for each substrate (corticosterone: 0.083, D8-corticosterone: 0.080, $p = 0.13$).

Figure 4-7. Tracer validation: metabolism of corticosterone and D8-corticosterone by 5β-reductase/3α-HSD physiological concentration range

Corticosterone (○) or D8-corticosterone (●) 20-300 nM incubated for 120-300 minutes with human hepatic cytosol (100 µg protein) and $[^3H]_4$-corticosterone (5 nM). N = 3, mean ± SEM.

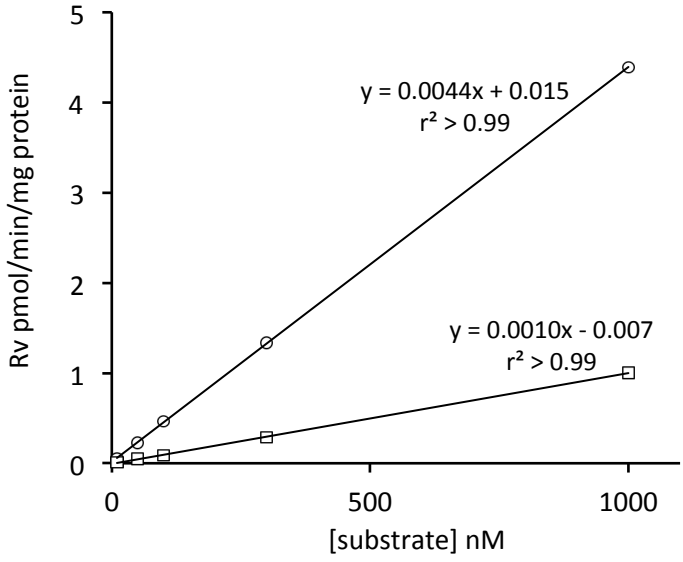


4.3.1.2. Metabolism of glucocorticoids by 5β-reductase/3α-HSD: corticosterone vs cortisol

The metabolism of cortisol by 5β-reductase/3α-HSD was compared to that of corticosterone within the physiological range of both glucocorticoids. For both substrates, non-saturable enzyme kinetics were observed (Figure 4-8), but the gradient of the regression line for corticosterone was greater than that for cortisol (by a factor of 4.3), indicating rate of metabolism of corticosterone exceeded that of cortisol for a given substrate concentration.

Figure 4-8. Metabolism of corticosterone and cortisol by 5β-reductase/3α-HSD

Corticosterone (○) or cortisol (□) incubated in duplicate for 60-240 minutes with human hepatic cytosol (200 μg protein) and $[^3H]_4$-labelled substrate (5 nM).



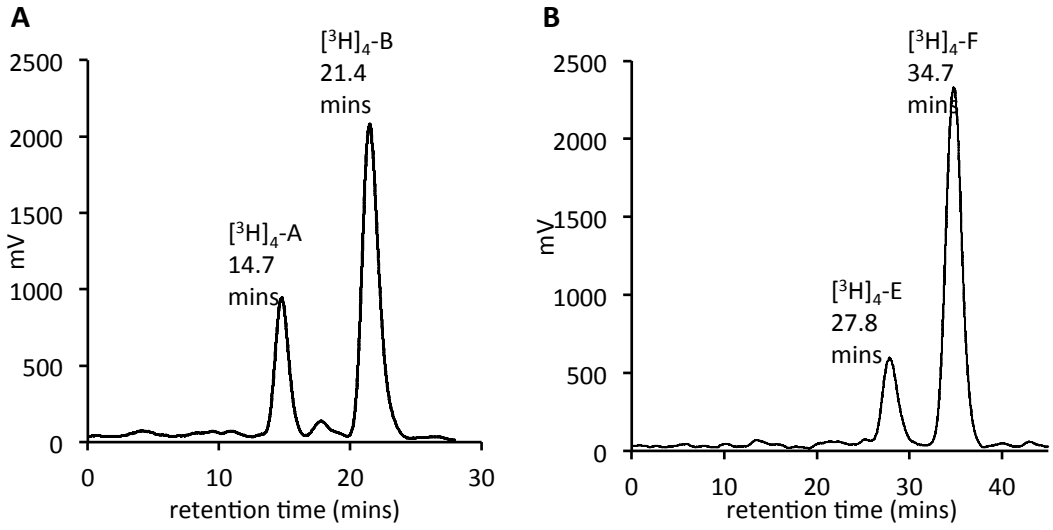
4.3.2. Metabolism of glucocorticoids by 11β-HSD2

Representative radio-chromatograms demonstrating the metabolism of $[^3H]$-labelled corticosterone and cortisol by HEK 293 cells stably transfected with 11β-HSD2 are shown in Figure 4-9. $[^3H]_4$-products were not formed in control samples without

cells or substrate. Following time-course experiments, an incubation time of 30 minutes was chosen to ensure reaction velocity remained linear and conversion of substrate to product was between 10 and 40 %. The identity of the products were confirmed by comparison of their retention times with those of $[^3H]_4$-standards.

Figure 4-9. Metabolism of $[^3H]_4$-corticosterone and $[^3H]_4$-cortisol by 11β-HSD2: representative chromatograms

Representative radio-chromatograms from extracts of cell-culture medium following incubation of $[^3H]_4$-corticosterone (panel A) or $[^3H]_4$-cortisol (panel B) with 5 x 10^5 HEK 293 11β-HSD2 cells, demonstrating conversion of corticosterone (B) to 11-dehydrocorticosterone (A); and cortisol (F) to cortisone (E).



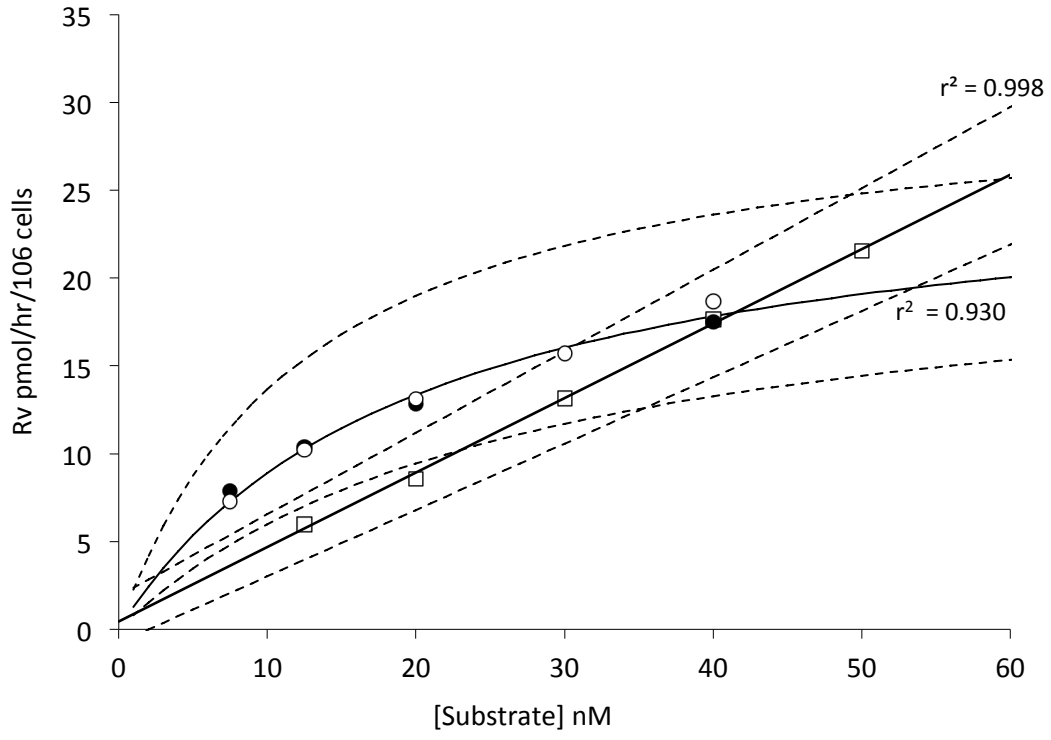
Both corticosterone and D8-corticosterone were substrates for human 11β-HSD2. Reaction velocity increased asymptotically with increasing substrate concentrations according to Michaelis-Menten kinetics (Figure 4-10). No statistically significant differences were found between modelled kinetic parameters for corticosterone (V_{max} = 28.6 ± 2.3 pmol/hr/10^6 cells; K_m = 22.9 ± 3.9 nM) and D8-corticosterone (V_{max} = 24.7 ± 3.6 pmol/hr/10^6 cells; K_m = 17.2 ± 5.5 nM; p = 0.67). Pooling both substrates

resulted in the following best-fit kinetic parameters (Figure 4-10): V_{max} = 26.7 ± 2.1 pmol/hr/10^6 cells; K_m = 20.0 ± 3.4 nM.

Metabolism of $[^3H]_4$-cortisol increased linearly with increasing substrate concentration ($r^2 > 0.99$). Direct comparison of the kinetic properties of the enzyme for cortisol and corticosterone was not possible due to the different kinetic models employed for each substrate. However, reaction velocities were broadly similar for a given substrate concentration within the range studied.

Figure 4-10. Metabolism of corticosterone, D8-corticosterone and cortisol by 11β-HSD2

HEK293 11β-HSD2 cells (5 x 10^5) incubated (30 mins) in duplicate with 2.5 – 35 nM corticosterone (○), D8-corticosterone (●) or cortisol (□) and corresponding $[3H]_4$-substrate (2.5 nM). Dashed lines denote 95 % confidence bands.



4.4. Discussion

Assessment of the *in vitro* metabolism of the stable isotope tracer D8-corticosterone has demonstrated that, across the range of concentrations likely to be relevant to human health and disease, the tracer is metabolised similarly to endogenous corticosterone. Use of the tracer *in vivo* is therefore unlikely to be confounded by a deuterium isotope effect, meaning valid observations regarding the endogenous compound can be made from such studies.

For human hepatic 5β-reductase/3α-HSD, a deuterium isotope effect was observed at supraphysiological substrate concentrations. It is likely that the effects seen in the above data are due to a secondary rather than primary isotope effect, since reduction of the steroidal A-ring occurs at the C=C bond adjacent to the deuterium at position 4 and not at deuterium-hydrogen bond itself. The magnitude of effect is consistent with a secondary deuterium isotope effect, where k_H/k_D is usually < 1.5 (Carey et al, 2008).

The data also reveal the metabolism of corticosterone and D8-corticosterone by human hepatic cytosol is subject to substrate inhibition, but that this phenomenon is also confined to supraphysiological concentrations. Similar observations have been made for other steroids, including testosterone (Di et al, 2008) and aldosterone (Chen et al, 2011), and are thought to be due to the presence of an alternative non-productive binding pocket within human 5β-reductase (Faucher et al, 2008) which accommodates steroids with a short C-17 constituent and prevents access of substrate to the active binding site.

Within the physiological range of concentrations for both cortisol and corticosterone, however, rate of catalysis shows an effectively linear response to increasing substrate concentration, suggesting the enzyme does not become saturated when glucocorticoid concentrations are relatively high, such as during the stress response. Under these circumstances, changes in clearance may occur in the absence of enzyme saturation, due to altered expression of the enzyme (Boonen et al, 2013) and potentially changes in hepatic blood-flow. Our kinetic findings are are consistent with those reported by others in which the K_m of 5β-reductase for cortisol is within the micromolar range (Iyer et al, 1990; Chen et al, 2011). However, the rate of metabolism of corticosterone by human 5β-reductase/3α-HSD is approximately 4-fold greater than that of cortisol.

The current data reflect the metabolism of active glucocorticoids by 5β-reductase and 3α-HSD in combination, and do not enable conclusions to be drawn regarding each reaction in isolation (although the intermediate metabolite dihydrocortisol was detected, its rate of formation was not linear over the incubation periods studied). The combined action of both enzymes on cortisol and corticosterone has not previously been assessed, but when purified human 5β-reductase has been studied in isolation (Chen et al, 2011), findings also suggest the enzyme metabolises corticosterone more efficiently than cortisol (k_{cat}/K_m = 0.87 vs 0.21 min^{-1} μM^{-1}).

Inactivation of cortisol by 11β-HSD2 is a second major pathway for glucocorticoid clearance, reflected in urinary excretion rates of 11-keto metabolites which are broadly similar to those of 11-hydroxyl metabolites (Andrew et al, 1998; Baudrand et al, 2011; Arlt et al, 2011). The current data are consistent with previous studies

carried out in tissue homogenates, which suggest the K_m of 11β-HSD2 for corticosterone is within the physiological range (low nanomolar) of the substrate (Albiston et al, 1994; Stewart et al, 1994; Gong et al, 2008). Reaction velocity continued to increase linearly with cortisol concentration beyond the K_m for corticosterone, indicating that should saturable kinetics occur for cortisol, the K_m exceeds that of corticosterone. This finding is also consistent with the above studies, which suggest the K_m of 11β-HSD2 for cortisol is approximately one order of magnitude greater than that for corticosterone. To assess the interconversion of 11-hydroxy and 11-keto steroids fully would require similar experiments to be performed using 11β-HSD1, but the current data suggest that both glucocorticoids are metabolised by 11β-HSD2 in a similar manner, in that the enzyme is unlikely to be saturated by either substrate under normal circumstances.

In the absence of significant differences in the proportion of free to bound substrate (Stroupe et al, 1978; Dunn et al, 1981), a proportionately higher affinity of 5β-reductase/3α-HSD for corticosterone suggests its hepatic extraction ratio, and therefore clearance, exceeds that of cortisol. Clearance also reflects the metabolism of glucocorticoids by processes not assessed in the current study, including oxidative removal of the C20, C21 side chain and 6β-hydroxylation, although these processes do not make a major contribution to total rates of clearance in healthy individuals (Sandberg et al, 1957; Voccia et al, 1979).

In conclusion, *in vitro* data suggest human hepatic enzymes clear corticosterone approximately 4-fold more rapidly than cortisol. In order to assess the *in vivo* metabolism of corticosterone, use of the stable isotope tracer

2,2,4,6,6,17α,21,21-[^{2}H]$_{8}$-corticosterone is proposed. Metabolism of this tracer reflects metabolism of the tracee. The following chapter (Chapter 5) discusses the development of an assay to quantify the deuterium labelled tracer and endogenous compound, in order for its use to be taken forward into pharmacokinetic (Chapter 6) and pharmacodynamic (Chapter 7) studies.

Chapter 5: LC-MS/MS analysis of D8-corticosterone

5.1. Introduction and Aims

The aim of the work presented in the current chapter was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to quantify the deuterium labelled compound 2,2,4,6,6,17α,21,21-[^{2}H]$_{8}$-corticosterone (D8-corticosterone) in human serum.

5.2. MS/MS optimisation

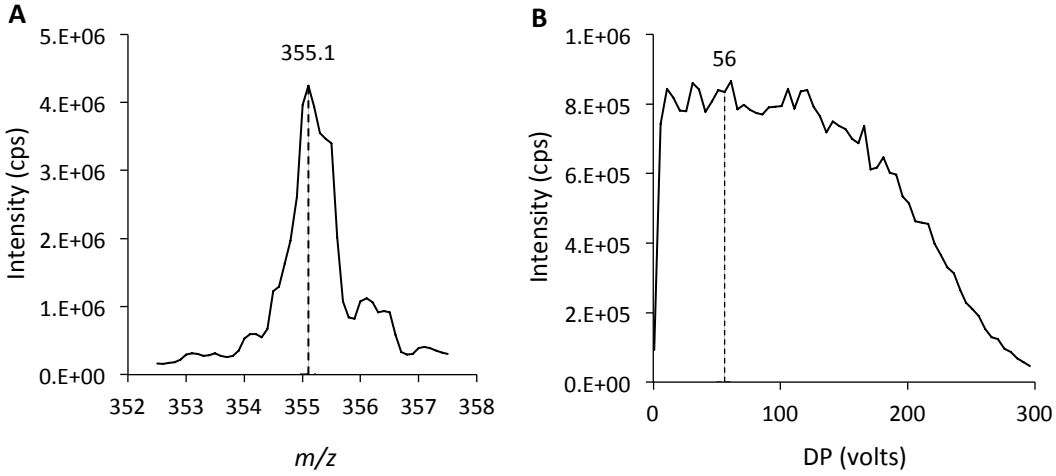
The assay was developed on a QTRAP® 5500 triple quadrupole mass spectrometer (Ab Sciex, Framingham, MA) using atmospheric pressure chemical ionisation (APCI) in positive ionisation mode (500 °C). Nitrogen was used as source and curtain gas (40 psi).

5.2.1. Q1 MS

A full scan (Q1) was undertaken (*m/z* 353–357) to confirm the presence of the expected $[M+H]^{+}$ ion for D8-corticosterone (average MW 354.46). The precursor ion, selected based upon maximal intensity at *m/z* 355.1 (Figure 5-1A) was analysed in Q1 selected ion monitoring (SIM) mode while varying voltage was applied to the orifice plate to determine optimal declustering potential (DP; Figure 5-1B). Default entrance potential for positive ions was chosen (10 V).

Figure 5-1. D8-Corticosterone Q1 MS

Panel A: Full scan (Q1) mass spectrum (*m/z* 353-357) confirming presence of expected protonated molecule (*m/z* 355.1). Panel B: Q1 selected ion monitoring (SIM; *m/z* 355.1) performed with varying declustering potential (DP). Dashed lines indicate parameters selected for subsequent MRM optimisation.



5.2.2. MRM (multiple reaction monitoring) optimisation

The precursor ion (*m/z* 355.1) was isolated by SIM (Q1) using the voltages determined above, and a full scan of Q3 (product ion scan) was performed to determine optimal MRM transitions (Figure 5-2A) following collision-induced dissociation (nitrogen gas; 2.6 x 10^{-5} Torr). The four most abundant product ions were selected for MRM mode, and collision energy (CE) and collision cell exit potential (CXP) were optimised for each transition by monitoring the signal intensity produced across a range of applied voltages (Figure 5-2B and C). The final product ion for quantitative analysis (*m/z* 337.2), corresponding to loss of water from the $[M + H]^+$ ion, was selected based upon maximal abundance using optimised parameters (Figure 5-2D).

The above procedure was also applied to unlabelled corticosterone to determine MRM transitions and voltages for quantitation by MS/MS. In the clinical studies described in Chapter 6 and 7, quantification of a number of other steroids was required. Optimal MS parameters for these analytes (previously established in-house), together with those for corticosterone isotopologues, are summarised in Table 5-1.

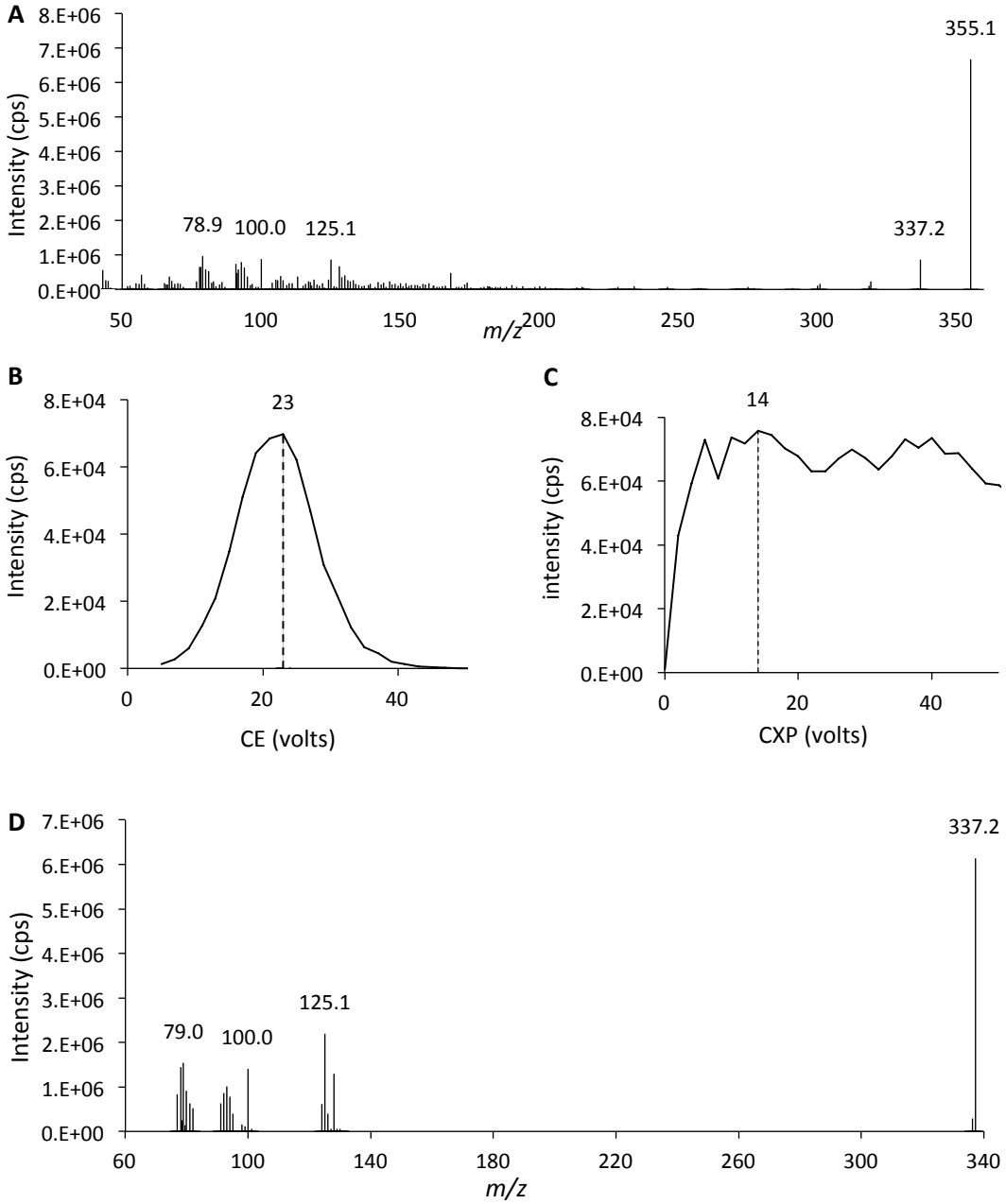
Table 5-1. Analysis of corticosterone, cortisol and deuterated isotopologues by LC-MS/MS: compound dependent MS parameters

Compound dependent parameters for simultaneously quantified compounds listed comprise ions selected in Q1 and Q3, dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP).

Compound	Q1 *m/z*	Q3 *m/z*	Time (msec)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
D8-corticosterone	355.147	337.000	250	116.000	10	19.000	14.000
Corticosterone	347.136	91.100	100	66.000	10	69.000	8.000
Cortisol	363.203	121.000	100	131.000	10	29.000	14.000
Epi-cortisol	363.203	77.000	100	141.000	10	101.000	14.000
D3-cortisol	366.198	121.200	100	121.000	10	27.000	20.000
D4-cortisol	367.017	121.100	100	121.000	10	25.000	20.000

Figure 5-2. D8-corticosterone MRM optimisation

Panel A: product ion scan mass spectrum (precursor ion *m/z* 355.1). Labels refer to the 4 most abundant product ions detected. Panels B and C: MRM (*m/z* 355.1-337.2) performed with varying collision energy (CE; panel B) and collision cell exit potential (CXP; panel C). Dashed lines indicate parameters selected for quantitative analysis. Panel D: repeat product ion scan (*m/z* 77.0 – 337.2) using optimised CE and CXP.



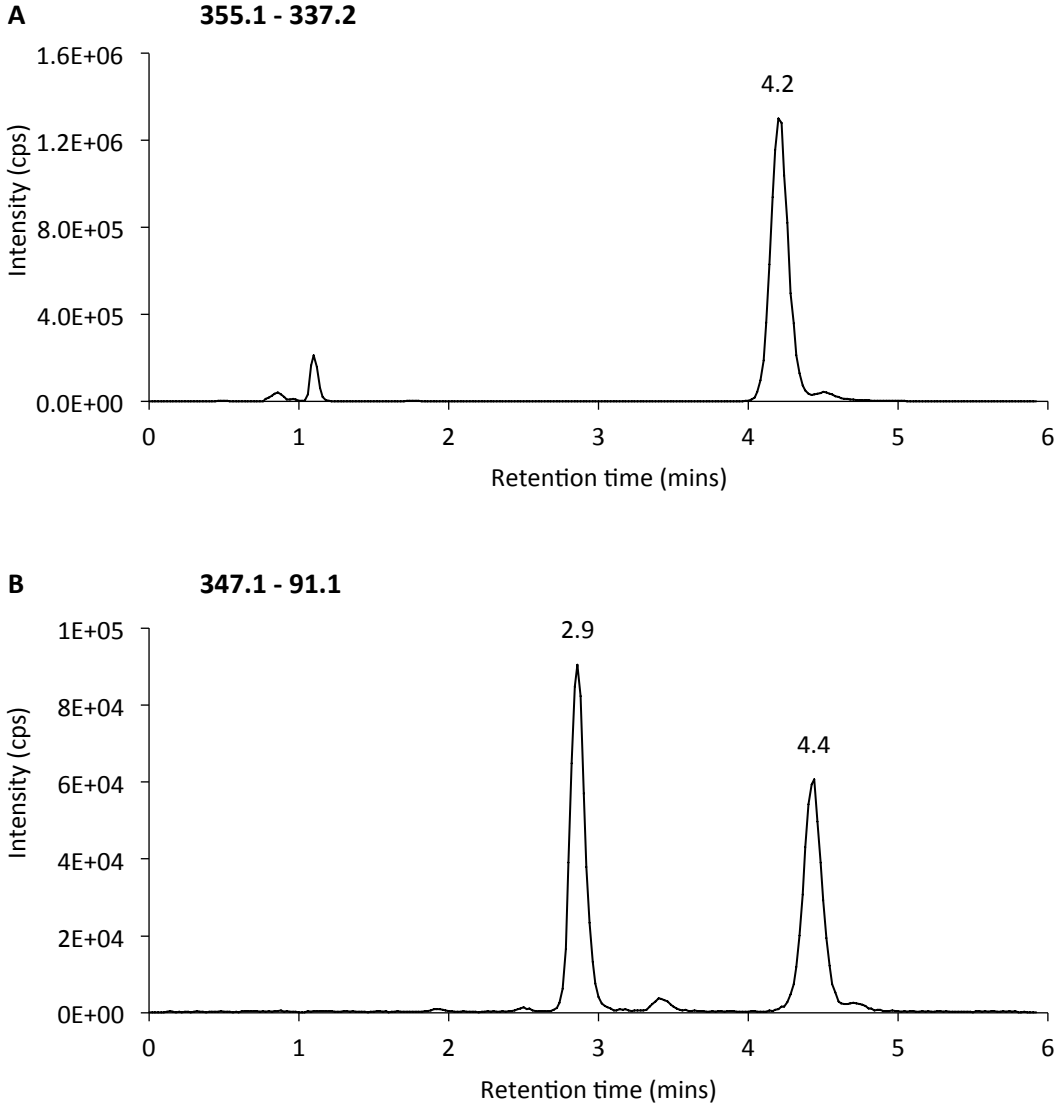
5.3. Liquid chromatography

Sample injection and chromatography was performed on the Acquity ultra performance liquid chromatography platform (UPLC®; Waters, Milford, MA). During assay development, chromatographic separation was achieved by gradient elution using a pentafluorophenyl (PFP) HPLC column (XSelect HSS PFP 100Å, 2.5 µm, 3 mm x 100 mm; Waters) with a total chromatographic run time of 9.0 minutes. To reduce run time, the assay was transferred to a UPLC column (Acquity UPLC HSS PFP 100Å, 1.8 µm, 2.1 mm x 50 mm; Waters), with a run time of 3.2 minutes. For the final method, which required quantification of multiple endogenous and deuterated compounds, a run time of 6.0 minutes was required using an octadecyl carbon (C18) column (SunFire, 100Å, 3.5 µm, 2.1 mm x 100 mm; Waters), using an isocratic method to separate compounds by reverse-phase chromatography. Sample (15 µl) was injected onto the column and steroid eluted with mobile phase (70 % water, 30 % acetonitrile; 0.1 % formic acid; flow rate 0.5 ml/min; temperature 10 °C).

Representative mass chromatograms (347.1 - 91.1 and 355.1 - 337.2) for corticosterone and D8-corticosterone are shown in Figure 5-3.

Figure 5-3. Representative mass chromatograms of D8-corticosterone (panel A); and corticosterone/epi-corticosterone (panel B) analysed as their $[M+H]^+$ ions by LC-MS/MS

Representative mass chromatograms of ion transitions 355.1 - 337.2 (panel A) and 347.1 - 91.1 (panel B) corresponding to fragmentation of [M+H]+ ions for D8-corticosterone and corticosterone/epi-corticosterone respectively (standards prepared in stripped serum processed as described in section 5.4; mass on column = 30 ng for each analyte). Labels refer to approximate retention times.

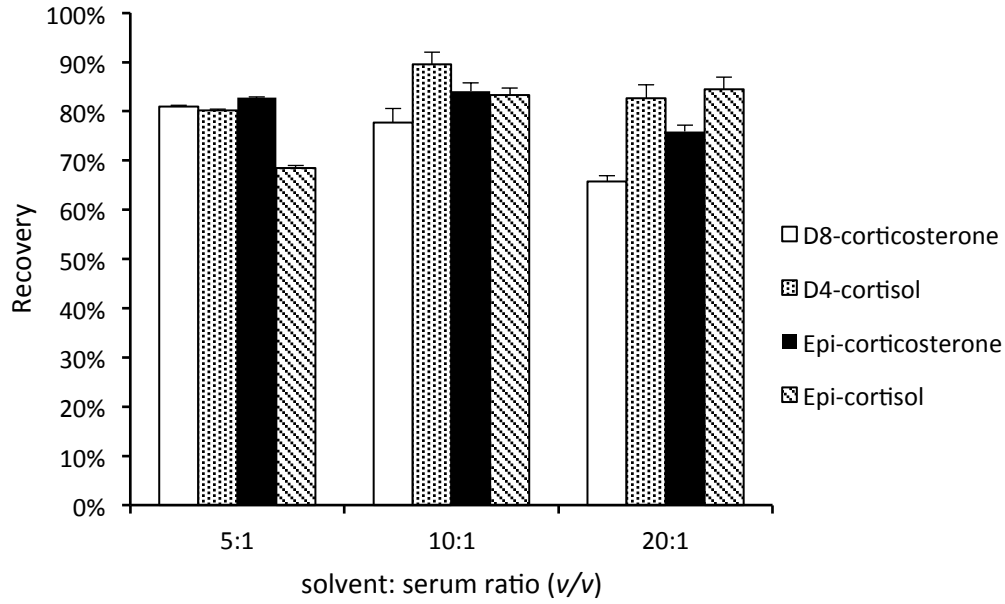


5.4. Extraction of glucocorticoids from serum samples and standards

In 16 × 150 mm glass culture tubes, serum was enriched with internal standard (epi-corticosterone; 20 ng (Chapter 6) or 200 ng (Chapter 7), dissolved in 10 - 20 µl methanol). Glucocorticoids were extracted from serum using a liquid-liquid extraction, which was optimised by assessing recovery of serum spiked with various steroids using different solvents and sample: solvent ratios (Figure 5-4). Ethyl acetate extraction produced a visibly contaminated organic phase, whereas extracts following chloroform extraction were clear. Following addition of chloroform (10 volumes), tubes were capped and mixed thoroughly. Excess plasma and lipid were removed using a glass pipette and discarded. The chloroform layer containing steroids was decanted into a clean 13 × 100 mm glass culture tube, and then transferred into a second clean glass tube before drying under 60 ºC OFN. Steroidal extracts were dissolved in mobile phase (70 % water, 30 % acetonitrile, 0.1 % formic acid *v/v*; 100 µl), transferred to disposable microcentrifuge tubes containing filter units (Spin-X, pore size 0.22 µm, Corning, San Nicolás, Mexico) and centrifuged (6000 rpm, 3 mins). The filtrate was transferred to auto sampler vials and either stored (-20 °C) or analysed immediately by LC-MS/MS (injection volume 15 µl).

Figure 5-4. Recovery of steroids from serum using chloroform extraction

Human serum (900 µl) was spiked with D8-corticosterone, D4-cortisol, epi-corticosterone and epi-cortisol (200 ng) in triplicate, and recovery assessed vs unextracted standards. A 10: 1 ratio (*v/v*) of chloroform: serum was chosen, which resulted in recovery of 78 ± 3 % for D8-corticosterone; 90 % ± 2 % for D4-cortisol; 84 % ± 2 % for epi-corticosterone and 83 % ± 1 % for epi-cortisol. Data refer to mean ± SEM.



5.5. Quantitative analysis

5.5.1. Source of serum for samples and standards

Serum was obtained during the clinical studies detailed in chapters 6 and 7. Commercially obtained stripped serum was used for standards and assay validation. Serum was thawed at room temperature prior to extraction.

5.5.2. Preparation of standard solutions

From stock solution (1 mg/ml in methanol), standard solutions were prepared in 12 × 75 mm glass culture tubes containing varying concentrations corticosterone and

D8-corticosterone in methanol. From these, 20 µl was transferred to 600 µl charcoal-stripped human serum to give the total mass of analyte required (0.15 - 10 ng per standard for clinical studies in Chapter 6 and 1 - 200 ng per standard for Chapter 7). Aqueous standards were also prepared (containing 10 ng analyte) to confirm retention times and instrumental sensitivity.

5.5.3. Sample volume

A sample volume of 600 µl was used for assay validation and in the bolus pharmacokinetic study (6.2.6). Because data derived from serum samples were not consistent with expected sensitivity derived from analysis of stripped serum standards (section 5.6.3), the assay volume was increased (1 ml) in subsequent clinical studies.

5.5.4. Data analysis

Compounds were quantified using Analyst software (version 1.6, AB SCIEX). The area under the peak of interest was integrated and expressed as a ratio of the area under internal standard (epi-corticosterone). Peak area ratio (y axis) was plotted against mass of steroid (x axis), and a line of best fit was drawn in the form $y = mx + c$.

Figure 5-8A and Figure 5-8B show representative standard curves for corticosterone and D8-corticosterone, respectively. Weighting was adjusted if necessary to optimise reproducibility of quantitation of low concentrations. Interpolation using the line of best-fit equation was used to determine steroid concentrations of serum samples. Controls containing mobile phase only were included at the start and end

of each batch of standards or unknowns to ensure carry-over of analyte did not arise between adjacent samples.

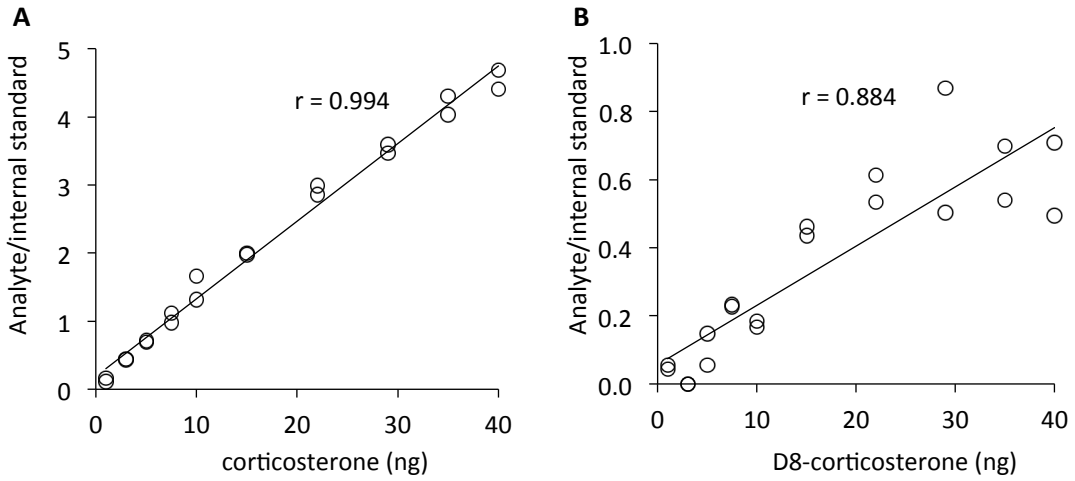
5.6. Assay Validation

5.6.1. Linearity

A linear response was defined by a regression coefficient (r) value of >0.99 across the range of expected serum concentrations. In repeated experiments (n = 3) during development of the assay in aqueous standards, a linear response was readily demonstrated for unlabelled corticosterone, but could not be demonstrated for D8-corticosterone under identical experimental conditions (See Figure 5-5 for representative data).

Figure 5-5. Linearity of response in LC-MS/MS analysis of corticosterone and D8-corticosterone

Methanolic standards prepared in duplicate containing analyte (0 – 40 ng) and internal standard (epi-corticosterone; 20 ng) were reduced to dryness; dissolved in mobile phase (50 % water, 50 % methanol, 0.1 % formic acid *v/v*; 100 µl); separated on an XSelect HSS PFP 2.5 µm, 3 mm x 100 mm column; and analysed by MS/MS (section 5.2.1).

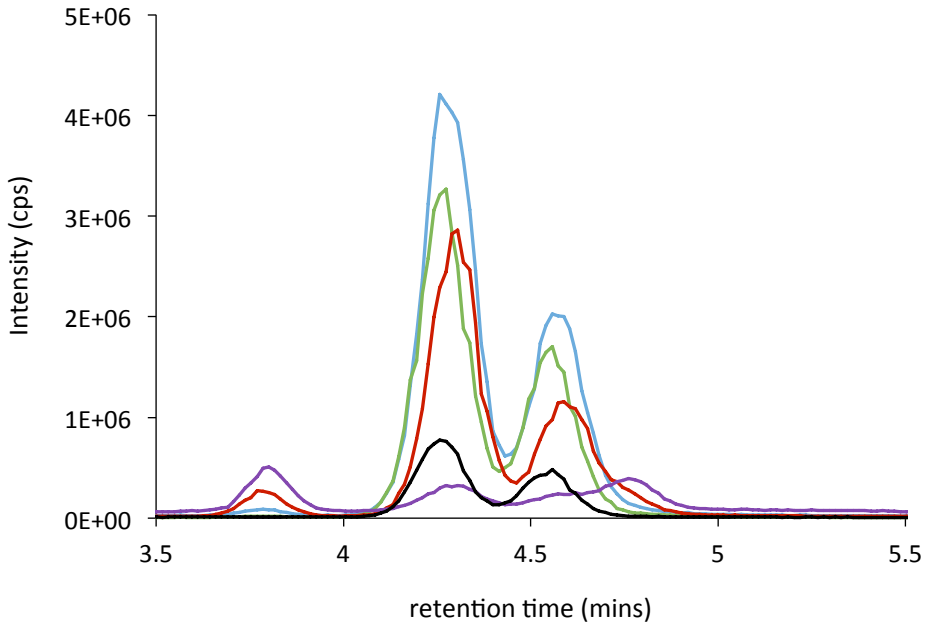


In troubleshooting of the assay, loss of deuterium from the D8-corticosterone tracer was identified. Deuterated compounds are commonly used as internal standards

because they commonly display similar extraction recovery, chromatographic retention times and ionisation efficiency to the analyte of interest. However, instability of the compounds is a recognised problem, reflected in loss of deuterium in exchange for hydrogen and reduced or absent signal intensity (Chavez-Eng et al, 2002; Stokvis et al, 2005). D-H exchange was confirmed by performing Q1 MS in SIM mode using *m/z* ratios which corresponded to the protonated products of D-H exchange from the D8-labelled compound (Figure 5-6).

Figure 5-6. Mass chromatograms for LC-MS/MS analysis of deuterated corticosterone isotopologues as their [M + H] ions, demonstrating deuterium/hydrogen (D-H) exchange.

Data are from representative serum sample from the clinical study described in Chapter 7. Q1 selected ion monitoring (SIM) for *m/z* ratios corresponding to protonated products of D-H exchange from D8-corticosterone ($[M+H]^+$ 355.1, black line) occurring at number of positions indicated by coloured lines: 1 (green), 2 (blue), 3 (red) and 4 (purple).



To attempt to stabilise deuterium labels on the compound, methoxime-trimethylsilyl (MO-TMSi) derivatives were formed and analysed by electrospray ionisation

GC-MS/MS as described in section 2.3.7.3. However, this technique could not be optimised to achieve sensitivity required for quantification of the compound at the concentrations required.

Linearity was achieved (Figure 5-8) in the final LC-MS/MS method employed to quantify the tracer in the presence of D-H exchange comprised summing peak areas corresponding to $[M + H]^+$ corticosterone ions labelled with 4 - 8 deuteriums.

5.6.2. Ion suppression

Ion suppression describes the phenomenon of reduced ionisation efficiency as a result of endogenous or exogenous compound(s) in the sample matrix which co-elute with the analyte of interest and compete for ionisation efficiency in the ionisation source (Annesley, 2003). Although ion suppression is more frequently a problem during electrospray ionisation, it can occur in APCI during the transfer of charge from the corona discharge needle to the analyte.

To investigate whether ion suppression occurs in the analysis of D8-corticosterone by LC-MS/MS, detector response (analyte peak area) was assessed in sample matrix (mobile phase prepared from human serum (1 ml), processed as described in section 5.4) spiked with analyte (50 ng D8-corticosterone, D4-cortisol, epi-corticosterone and epi-cortisol) after processing). Comparison was made with matrix used in standards (human steroid-stripped serum) spiked post-processing, and spiked mobile phase that had not undergone processing.

5.6.2.1. Data analysis

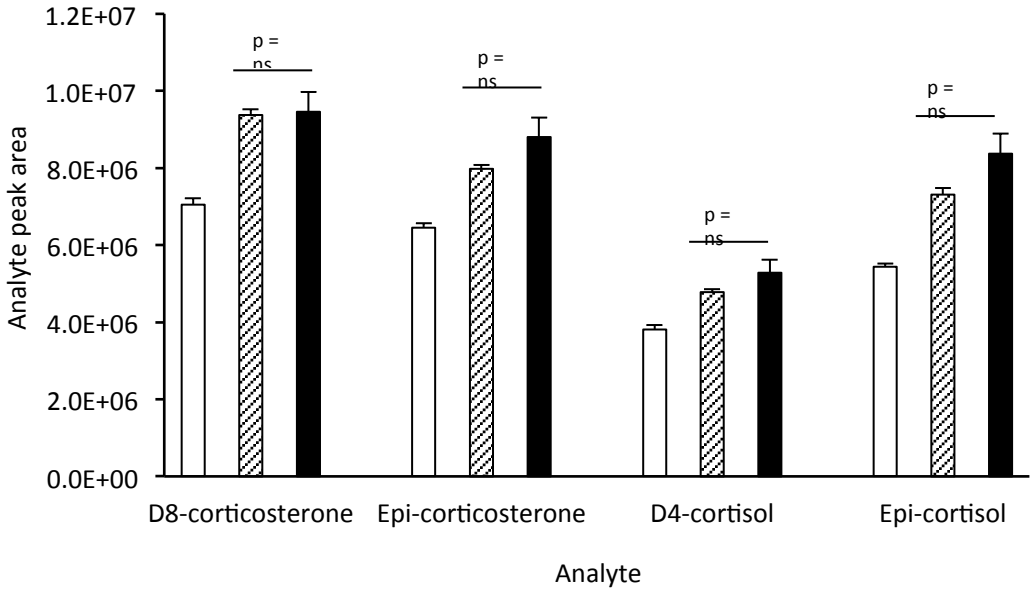
Comparison of analyte peak area between experimental matrices was undertaken by one-way ANOVA with Tukey's post hoc-test (GraphPad Prism V5.01, GraphPad Software Inc, La Jolla CA). Independent analyses were undertaken for each analyte.

5.6.2.2. Results

Analyte peak area in sample matrix (unstripped serum) did not differ to that in standard matrix (stripped serum) for any of the analytes studied (Figure 5-7). For all analytes, peak area was lower in aqueous standards in comparison to both biological matrices ($p < 0.05$, reflecting a degree of ion enhancement and/or increased background signal in serum vs aqueous samples. Therefore, stripped serum was used in all assays for preparation of standards to minimise matrix effects.

Figure 5-7. Analysis of glucocorticoids by LC-MS/MS: assessment of ion suppression

Comparison of analyte peak area following addition of analyte (50 ng; n = 3) to three different experimental matrices (1 ml): mobile phase (open bars); steroid-stripped serum following chloroform extraction (hatched bars); and serum which had not been stripped of steroids but which had undergone chloroform extraction (closed bars). Ns = not significant.



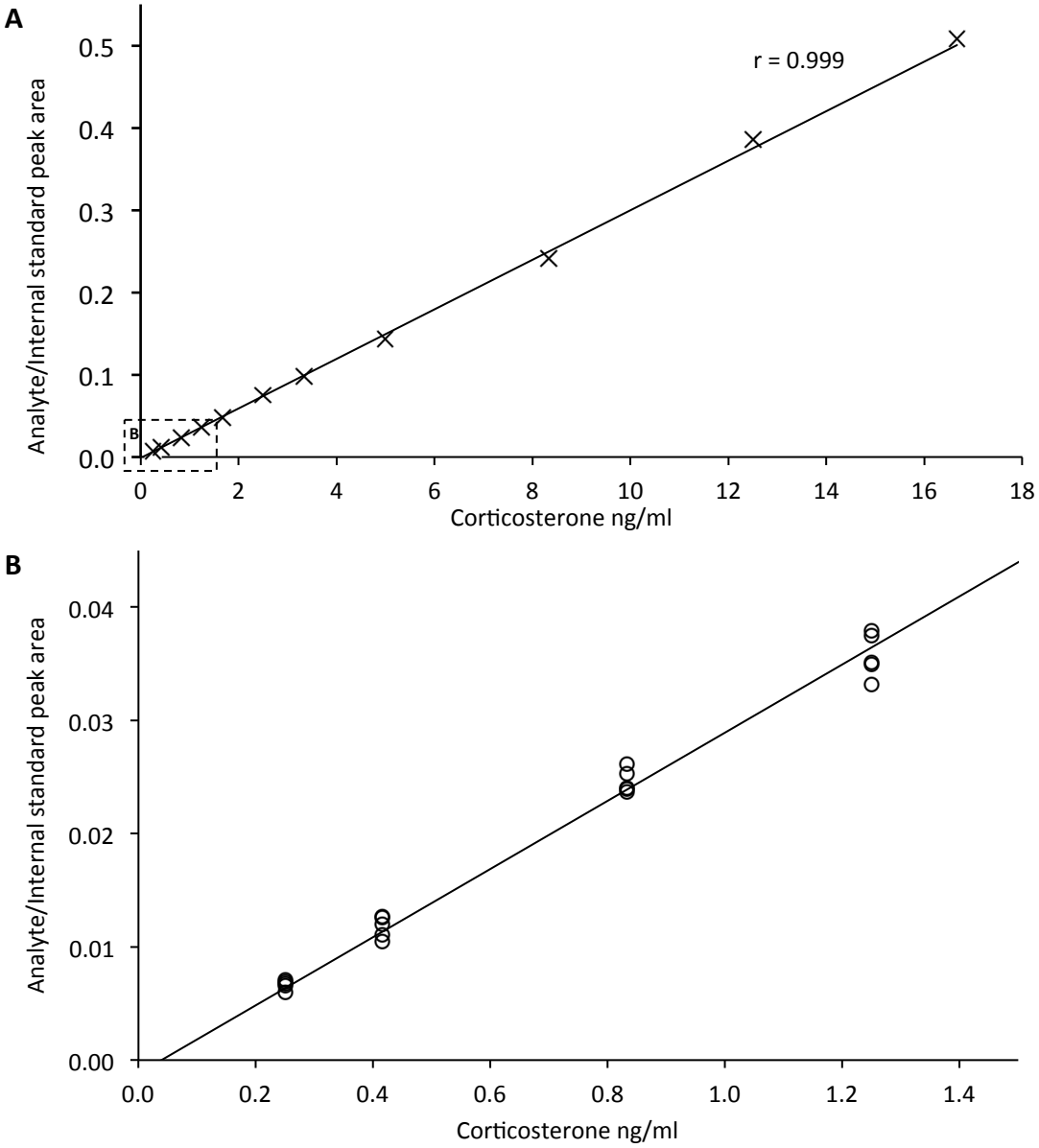
5.6.3. Limit of quantitation, accuracy and precision

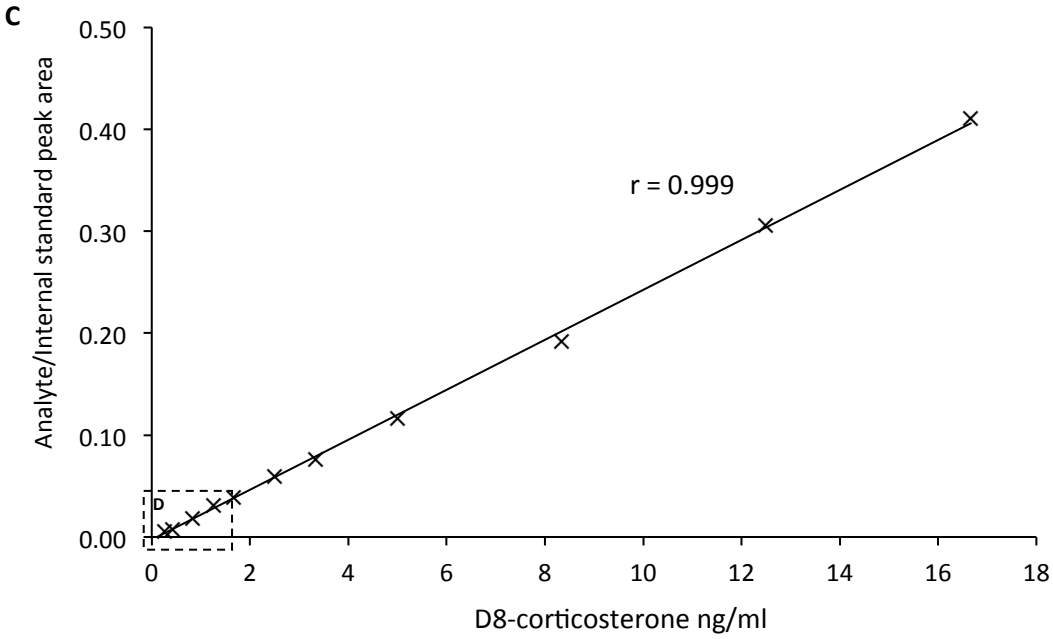
The limits of quantitation (LoQ) for corticosterone and D8-corticosterone were calculated by assessing the analyte/internal standard peak area in steroidal extracts (n = 6 at each concentration) from stripped human serum (600 µl) spiked with analyte and internal standard (epi-corticosterone 20 ng). The quantifiable range of the standard curve was defined as the range at which the coefficient of variation (CV) and accuracy of estimates was ≤ 15 % (20 % at the lower LoQ). Using these criteria, the LoQ for corticosterone was 0.25 ng/ml and for D8-corticosterone 0.42 ng/ml (Figure 5-8 and Table 5-2). Accuracy and precision were also confirmed to be within the above ranges in spike standards chosen to correspond to anticipated

mid-range (15 ng/ml; 43.3 nM) and high-range (35 ng/ml; 101 nM) serum samples from clinical studies.

Figure 5-8 and Table 5-2. Corticosterone/D8-corticosterone LC-MS/MS: accuracy, limit of quantitation (LoQ) and precision.

Standard curves in stripped serum spiked with corticosterone (panel A) and D8-corticosterone (panel C); internal standard epi-corticosterone. Standard curve (weighted 1/*x*) derived from a single standard at each point. Dashed box indicates range of concentrations chosen to determine lower limit of quantitation, detailed in (B & D): Replicates (n = 5) performed at 0.25, 0.42, 0.83 and 1.25 ng/ml (assuming 600 µl sample volume). Calculated concentrations, derived from standard curves A & C were used to determine accuracy and precision (Table 5-2).




C
0.50
0.40
0.30
0.20
0.10
0.00
Analyte/Internal standard peak area
r = 0.999
D
0
2
4
6
8
10
12
14
16
18
D8-corticosterone ng/ml

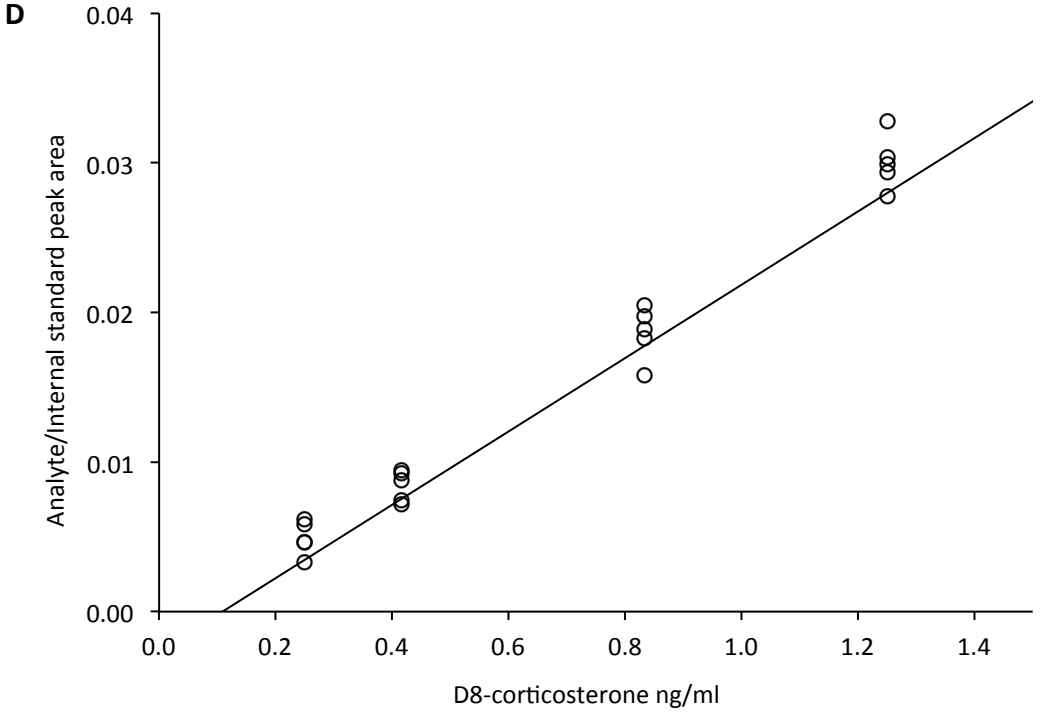

D
0.04
0.03
0.02
0.01
0.00
Analyte/Internal standard peak area
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
D8-corticosterone ng/ml

Table 5-2. Corticosterone/D8-corticosterone LC-MS/MS: accuracy, limit of quantitation (LoQ) and precision

Intra-assay co-efficient of variation (CV) determined from replicates of 5 as detailed in Figure 5-8 (with additional mid (3.33 ng/ml) and high (16.7 ng/ml) concentrations not shown in figures). RMSE = root mean squared error.

	Corticosterone			D8-corticosterone		
ng/ml	Accuracy		CV	Accuracy		CV
	RMSE	Range		RMSE	Range	
0.25	6.0 %	95.3 % - 109.6 %	5.3 %	14.2 %	78.8 % - 126.3 %	17.8 %
0.42	7.0 %	92.8 % - 110.6 %	7.5 %	8.4 %	85.2 % - 108.7 %	10.6 %
0.83	3.3 %	99.0 % - 105.8 %	4.2 %	6.3 %	85.8 % - 108.8 %	8.9 %
1.25	4.6 %	96.0 % - 105.8 %	5.4 %	5.5 %	96.0 % - 113.2 %	5.8 %
3.33	2.5 %	110.8 % - 105.3 %	1.7 %	4.1 %	96.3 % - 108.3 %	4.4 %
16.7	2.2 %	100.3 % - 106.1 %	2.3 %	2.7 %	99.1 % - 107.2 %	2.9 %

5.7. Discussion

We have developed and validated a LC-MS/MS assay to quantify corticosterone and the deuterium labelled tracer D8-corticosterone in human serum. Deuterium-hydrogen exchange affects the quantification of D8-corticosterone, and the assay has been adjusted to account for this. The assay is highly sensitive and unaffected by ion suppression in human serum, with a lower limit of quantitation of 0.25 ng/ml (0.7 nM) for corticosterone and 0.42 ng/ml (1.2 nM) for D8-corticosterone. Development and validation of the assay is a key requirement for subsequent clinical studies, which aim to assess D8-corticosterone pharmacokinetics (Chapter 6) and pharmacodynamics (Chapter 7).

Chapter 6: *In vivo* metabolism of corticosterone

6.1. Introduction and Aims

Data presented in Chapter 4 demonstrate the enzymes that catalyse the key steps in glucocorticoid clearance metabolise corticosterone more rapidly than cortisol. When clearance of substrate is greater, rate of production must also be greater in order to sustain a given substrate concentration. Therefore, the rate of turnover of corticosterone may be disproportionately high, despite the relatively low circulating concentration of the compound. Rapid turnover of substrate is expected to result in circulating concentrations that are highly sensitive to changes in rates of production and/or clearance. Turnover is therefore an important determinant of hormone action within a dynamic system such as the HPA axis.

In this chapter, the aim was to establish whether differential hepatic metabolism of corticosterone and cortisol demonstrated *in vitro* are reflected in higher clearance of corticosterone than cortisol *in vivo*. Two different approaches were employed to address these aims. Firstly, isotope dilution at steady state was assessed using the deuterium-labelled corticosterone tracer discussed in the previous two chapters. Data from these studies were used to design dosing protocols for the clinical study presented in Chapter 7. Secondly, production rates were assessed by analysis of urinary steroids by gas-chromatography mass tandem spectrometry (GC-MS/MS).

6.1.1. Aims

i. To establish the volume of distribution and half life of the stable isotope tracer D8-corticosterone following bolus injection in healthy male volunteers

ii. Using results from (i), to undertake primed steady state infusion, to establish rate of clearance and production of corticosterone and pilot optimal infusion rates for ACTH suppression (Chapter 7)

iii. To quantify normal daily urinary excretion rates of corticosterone and metabolites (17-deoxysteroids), and compare these to the excretion rates of cortisol and metabolites (17-hydroxysteroids)

6.2. Methods: D8-corticosterone pharmacokinetic studies

6.2.1. Ethical and Research Governance Approvals

Approvals were obtained from the South East Scotland Research Ethics Committee (11/SS/0046) and NHS Lothian Research and Development (2011/R/END/03). Research support approvals were obtained from the Wellcome Trust Clinical Research Facility (WTCRF), clinical laboratories, and radiopharmacy at the Royal Infirmary, Edinburgh (RIE).

6.2.2. Participants

Healthy male volunteers (bolus study: n = 3; steady state infusion study: n = 6) were recruited by advertisement at the University of Edinburgh.

6.2.2.1. Inclusion criteria

- Male
- Age 20-50 years
- Able to give informed consent
- No major illnesses
- BMI 20-25 kg/m^2
- No regular medication
- Alcohol intake < 28 units per week
- Normal full blood count (FBC), urea and electrolytes (UE), liver function tests (LFT), thyroid function tests (TFT), and blood glucose.

6.2.2.2. Exclusion criteria

- Glucocorticoid treatment within the previous 3 months by any route

- Blood donation within the preceding 3 months
- Research study participation within the preceding 6 weeks

6.2.3. Clinical measurements

6.2.3.1. Measurement of standing height

Standing height was measured to the nearest 0.1 cm with volunteers stood (without shoes), feet parallel and head, shoulder blades and heels against the back of a wall mounted ruler.

6.2.3.2. Measurement of weight

Weight was measured to the nearest 0.1 kg using an electronic scale (Seca, Birmingham, UK) after volunteers removed outdoor clothing, shoes and heavy items from pockets.

6.2.3.3. Measurement of systolic and diastolic blood pressure

Blood pressure was measured after sitting for at least 10 minutes using a 705IT automatic blood pressure monitor (OMRON Healthcare Europe BV, Hoofddorp, NL).

6.2.4. Screening visits

Participants attended the WTCRF at RIE, where written informed consent was obtained, weight and height recorded, and a brief medical history documented to ensure no contraindications to participation were present. Blood samples were taken for FBC, UE, LFT, TFT, and glucose (analysed at clinical laboratories, RIE).

6.2.5. Study visits

Participants attended the WTCRF, RIE at 0830h following an overnight fast (from 2200h). Intravenous cannulae (18G) were inserted in each antecubital fossa using an aseptic technique. Blood pressure and pulse were recorded, and venous blood was collected for basal assessment of cortisol, D8-corticosterone and corticosterone. Beginning at 0900h, D8-corticosterone was administered through the cannula in the left arm, and blood samples collected according to the sampling protocol through the cannula in the right arm. At the end of the study visit, cannulae were removed, blood pressure and pulse were rechecked and participants provided with lunch. In the bolus study, participants attended for a repeat study visit after an interval of at least 2 weeks.

6.2.6. Bolus study

6.2.6.1. Preparation of the stable isotope tracer D8-corticosterone

D8-corticosterone was dissolved in pharmaceutical grade ethanol and filtered in the radiopharmacy to form a sterile stock solution, stored (-40 °C) for a maximum of 8 weeks. Solutions were defrosted and infusions prepared in the clinical research facility on the morning of each study.

6.2.6.2. Tracer administration protocol

D8-corticosterone stock solution was prepared in ethanol as above at 100 µg/ml (1 ml aliquots). For a single bolus dose, stock solution (0.5 ml) was added to sodium

chloride 0.9% *w/v* (49.5 ml) to give a final concentration 1 μg/ml. An intravenous bolus (50 μg) was administered over 5 minutes.

6.2.6.3. Sample collection and processing

Serum samples for D8-corticosterone were obtained in serum gel tubes (4.9 ml, Monovette®, Sarstedt, Nümbrecht, Germany). Samples were gently mixed by inverting several times, left for centrifugation (1912 × *g*, 10 min, 4 °C) to obtain serum, which was separated and stored (aliquots of 600 - 700 μl) at -80 ºC for analysis by LC-MS/MS (Chapter 5).

6.2.6.4. Sampling protocol

Samples were drawn at 2 minute intervals from $t = 0$ (time of beginning of bolus injection) until $t = 16$ mins, 5 minute intervals from $t = 20$ - 45 mins, and 15 mins from $t = 45$ - 180 mins.

6.2.7. Steady state study

Data from the bolus study were used to determine doses (section 6.2.8) for a primed steady state infusion in healthy male subjects (n = 6), recruited according to the methods and criteria described in section 6.2.2. We aimed for a target steady state concentration of 15 nM.

6.2.7.1. Tracer administration protocol

D8-corticosterone stock solution was prepared in ethanol (section 6.2.6.1) at 909 μg/ml (3 ml aliquots). For each study visit, stock solution (2 ml) was added to sodium chloride 0.9 % *w/v* (498 ml) to give a final concentration 3.64 μg/ml. An

intravenous bolus (76.7 µg) was administered over 3 minutes, followed by constant infusion (7.4 µg/min) until *t* = 180 mins. A sample of infusate (1 ml) from each study visit was retained in order to confirm the infused concentration of D8-corticosterone.

6.2.7.2. Sampling protocol

Samples were drawn at 15 minute intervals from *t* = 0 - 180 mins. Sample processing and analysis was undertaken as described in section 6.2.6 with samples stored in larger aliquots as compared to the bolus study (900 µl serum vs 450 - 600 µl). In addition to D8-corticosterone, LC-MS/MS analysis of endogenous corticosterone and cortisol was also undertaken in order to assess tracer enrichment and to gain preliminary data on the impact of tracer infusion on the normal diurnal profile of circulating glucocorticoid concentrations.

6.2.8. Pharmacokinetic analysis

Pharmacokinetic analysis was undertaken using Kinetica software (Adept Scientific, Letchworth Garden City, UK).

6.2.8.1. Bolus pharmacokinetic study

Pharmacokinetic parameters were derived according to the following equations:

Elimination rate constant (K_{el})

$$K_{el} = \frac{ln\,2}{t_{1/2}}$$

Volume of distribution (V_d)

$$V_d = \frac{dose}{C_0}$$

Where *dose* = bolus dose and C_0 = plasma concentration at $t = 0$ (peak plasma concentration was used as an approximation for C_0).

Clearance (Cl)

$$Cl = V_d \times K_{el}$$

6.2.8.2. Steady state pharmacokinetic study

Priming dose

The priming dose required for the target steady state concentration C_{ss} was calculated according to the following equation:

$$priming\ dose = C_{ss} \times V_d$$

Rate of infusion (R_i)

$$R_i = C_{ss} \times Cl$$

Where (Cl) = clearance estimated from bolus study (section 6.2.6)

Clearance (Cl)

$$Cl = \frac{R_i}{C_{ss}}$$

Volume of distribution (V_d)

$$V_d = Cl \times \frac{t_{1/2}}{\ln(2)}$$

Rate of appearance (R_a)

$$R_a = \frac{R_i}{t/_T}$$

Where $t/_T$ = tracer: tracee ratio

6.3. Urinary metabolism of corticosterone vs cortisol

6.3.1. Source of samples

In a recent randomised controlled trial conducted by the supervisors' group (Upreti et al, 2014), 24 hour excretion rates of 17-hydroxysteroids had previously been quantified in healthy male volunteers (age 20-85 years). Principal exclusion criteria were glucocorticoid use in the previous 3 months; diabetes mellitus or impaired glucose tolerance; significant hepatic, renal or thyroid disease; and BMI $\geq$ 40 kg/m^2. Each participant collected a 24 hour urine sample (beginning at the 2nd void), the volume of which was measured.

6.3.2. Methods

From aliquots (20 ml) of urine collected before administration of the study drug, steroids were extracted, derivatised and quantified (section 2.3.7). Repeat analysis (n = 24; chosen at random) of remaining organic extracts stored (-20 °C) from samples collected pre-randomisation was undertaken to extend the range of steroids quantified to include both 17-deoxy- and 17-hydroxy-steroids.

6.3.3. Data Analysis

Data were analysed using Xcalibur 2.1 software (Thermo Scientific Inc, Waltham, MA). In stored organic extracts used to quantify 24 hour urinary steroid excretion rates, original standard curves did not contain 17-deoxysteroids. Concentrations of these analytes were therefore calculated from a new standard curve spiked with the same mass of internal standard as the original samples. Validity of this approach

was assessed by assessing equivalence of original and new standard curves produced for 17-hydroxysteroids (RMS for difference in gradient = 10.9 %), and by comparing estimates of concentrations produced from each standard curve (Figure 6-3).

6.4. Results

6.4.1. Pharmacokinetic studies

Characteristics of participants in both pharmacokinetics studies are summarised in Table 6-1.

Table 6-1. Characteristics of participants in D8-corticosterone pharmacokinetic studies

Data refer to mean ± standard deviation

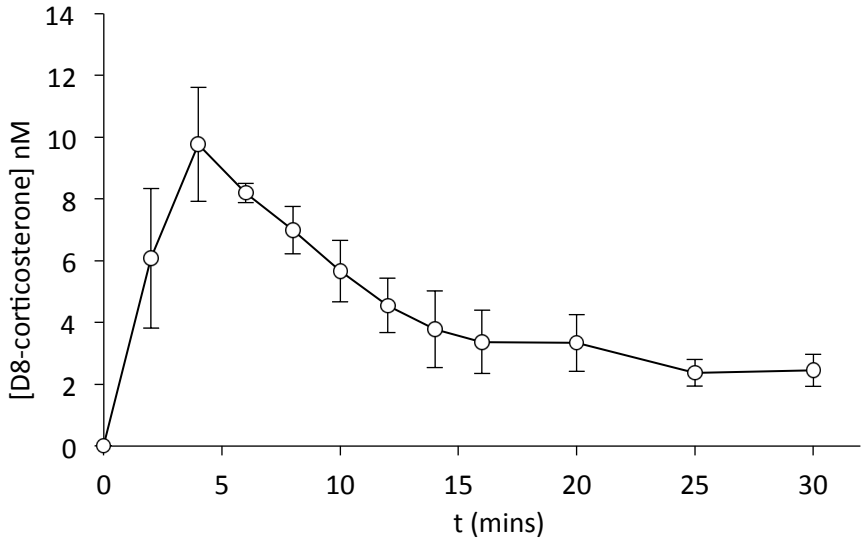
	Bolus study	Steady state study
n	3	6
Age (years)	24.0 ± 2.6	24.7 ± 5.6
BMI (kg/m^2)	23.4 ± 1.4	22.2 ± 1.3

6.4.1.1. Bolus pharmacokinetic study

A bolus dose of 50 μg D8-corticosterone resulted in a peak serum concentration (occurring at t = 4 mins) of 9.77 ± 1.85 nM (Figure 6-1) and estimated V_d 14.1 ± 1.6 litres. The tracer was rapidly eliminated, and was not quantifiable in samples beyond t = 30 mins. The decline in serum corticosterone concentration was modelled according to a single compartment model (K_{el} 0.121 ± 0.042 min^{-1}; t ½ 9.7 ± 3.3 mins). Clearance, derived from these parameters, was 1.46 ± 0.36 l/min.

Figure 6-1. D8-corticosterone bolus pharmacokinetics

n = 3, mean ± SEM



6.4.1.2. Steady state pharmacokinetic study

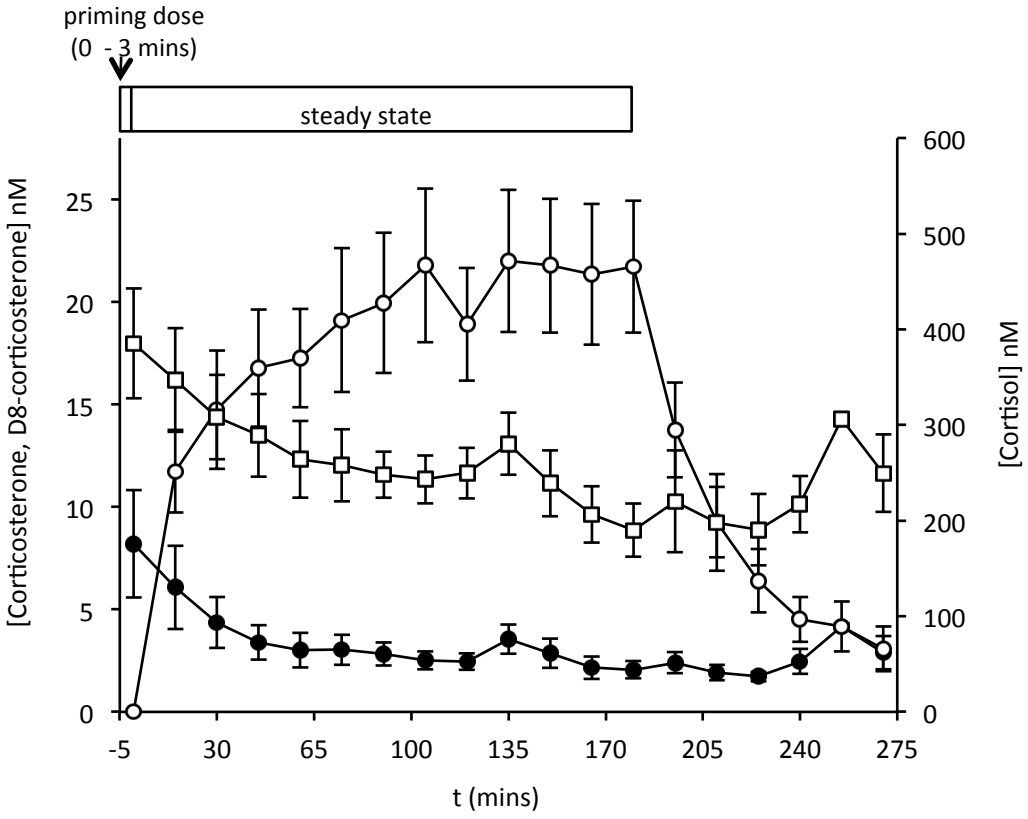
Measured infusate concentration was higher than anticipated concentration (measured 4.12 ± 0.13 μg/ml; calculated 3.64 μg/ml; CV 6.0 %). Data are derived from measured rather than calculated concentrations.

Preliminary analysis (n = 3) was performed while the study was underway in order to determine whether any adjustment to the infusion protocol was required. D8-corticosterone reached steady state at $t \sim = 105$ mins (Figure 6-2). The interval to steady state was longer than expected from the bolus pharmacokinetic data, and given the use of a priming dose. As a result, in the final 3 participants, an amendment was obtained to extend the sampling protocol by 90 mins (continuing at

15 min intervals) after discontinuing D8-corticosterone infusion, in order to re-estimate t ½.

Figure 6-2. D8-corticosterone steady state pharmacokinetics

n = 6 (n = 3 at t > 180 mins), mean ± SEM. D8 corticosterone (open circles); corticosterone (closed circles; cortisol (squares).



Mean C_{ss} (t = 105 - 180 mins) was 21.3 ± 0.5 nM. Over the same interval, mean tracer: tracee ratio was 9.7 ± 1.3, and corticosterone production rate (R_a) at steady state 0.49 ± 0.06 µg/min (1.42 ± 0.18 nmol/min). Calculated clearance was 1.1 ± 0.20 l/min. The decay in serum corticosterone concentrations following discontinuation of tracer infusion was modelled according to first-order kinetics,

with K_{el} 0.031 ± 0.014 min^{-1} and t ½ 28.5 ± 3.3 mins. Apparent V_d derived from clearance and half life in the steady state study was 45.7 ± 5.3 l.

Pharmacokinetic parameters derived from both studies are summarised in Table 6-2.

Table 6-2. Pharmacokinetics of D8-corticosterone (D8-B) and corticosterone (B) production rate during D8-B infusion

Data refer to mean ± SEM.

	Bolus study	Steady state study
Apparent volume of distribution (l)	14.1 ± 1.6	45.7 ± 5.3
Clearance (l/min)	1.46 ± 0.36	1.11 ± 0.20
Elimination rate constant (min^{-1})	0.121 ± 0.042	0.031 ± 0.014
Half life (min)	9.7 ± 3.3	28.5 ± 3.3
B rate of appearance (nmol/min)	n/a	1.42 ± 0.18

6.4.2. Urinary metabolites of corticosterone vs cortisol

Mean age (± standard deviation) of the study participants (n = 24) was 40.9 ± 21.0 (range 21-85). Mean BMI (± standard deviation) was 27.0 ± 3.0 (range 22.5-33.9). Excretion rates of urinary glucocorticoids over 24 hours are illustrated in Figure 6-3 and Table 6-3.

Figure 6-3 and Table 6-3 (over). Daily excretion rate of urinary corticosterone (B) metabolites (17-deoxysteroids) and cortisol (F) metabolites (17 hydroxysteroids) in male volunteers

Note log scale of y-axis. Steroid excretion rates derived from original analysis (Upreti et al, 2014)(hatched bars) and re-analysis/analysis of steroids not previously quantified (open bars & values in table). See over for abbreviations.

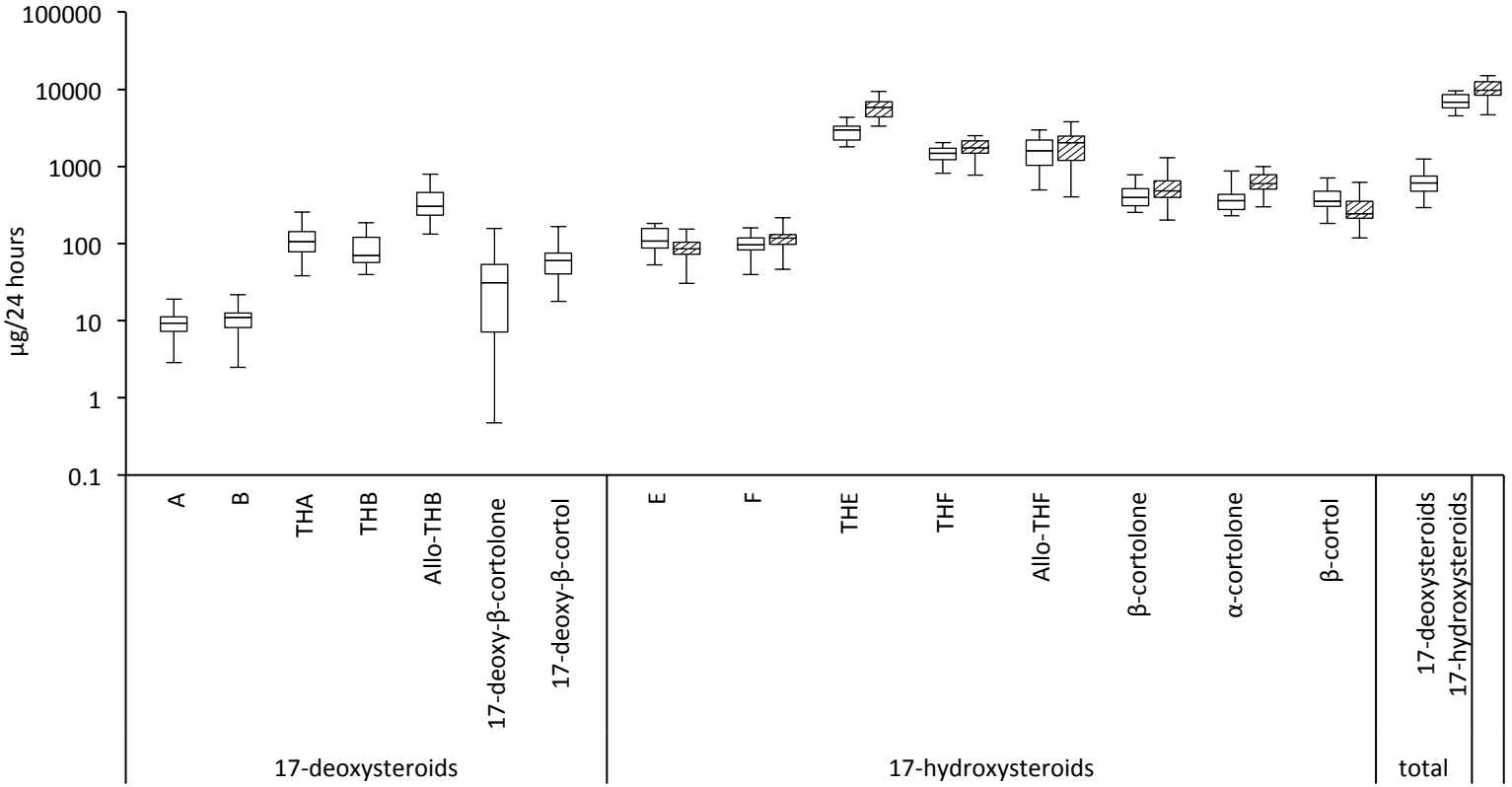


Table 6-3. Daily excretion rate of urinary corticosterone (B) metabolites (17-deoxysteroids) and cortisol (F) metabolites (17 hydroxysteroids) in male volunteers

A = 11-dehydrocorticosterone; THA = 11-dehydro-tetrahydrocorticosterone; THB = tetrahydrocorticosterone; E = cortisone; THE = 5β-tetrahydrocortisone; THF = 5β-tetrahydrocortisol; 5α-isomers designated 'allo'. Standards were not available for 17-deoxy-α-cortolone or 5α--THA; corresponding 17-hydroxylated steroids are not included in data for comparison of total 17 deoxy- and hydroxysteroid excretion.

		Median	Q1	Q3	Min	Max
17-deoxysteroids (µg/24 hours)	11-Dehydrocorticosterone	9.4	7.3	11.2	2.9	19.1
	Corticosterone	11.1	8.1	12.5	2.5	21.5
	THA	106.6	77.9	142.5	38.3	258.6
	THB	70.5	56.8	119.8	39.4	187.6
	Allo-THB	305.9	232.0	462.5	131.9	791.6
	17-deoxy-β-cortolone	31.1	7.1	53.5	0.5	157.3
	17-deoxy-β-cortol	60.0	40.8	75.9	17.8	167.6
17-hydroxysteroids (µg/24 hours)	Cortisone	107.3	86.9	156.8	53.0	183.1
	Cortisol	96.1	83.4	119.1	39.4	161.0
	THE	2989.1	2187.2	3305.5	1794.7	4297.9
	THF	1479.1	1223.2	1733.6	816.3	2033.7
	Allo - THF	1599.5	1024.3	2210.1	493.0	2995.1
	β-Cortolone	394.1	306.9	515.9	253.5	780.9
	α-Cortolone	357.9	278.0	435.9	229.5	868.7
	β-Cortol	354.2	301.7	474.8	182.0	704.5
Total (µg/24 hours)	17-deoxysteroids	615.4	476.5	744.2	291.2	1238.6
	17-hydroxysteroids	6844.1	5753.8	8600.5	4524.7	9627.5
	Ratio 17 deoxy/hydroxy	8.8%	7.7%	11.0%	5.4%	13.3%

6.5. Discussion

The half-life, clearance and volume of distribution of the stable isotope tracer D8-corticosterone following bolus injection and steady-state infusion have been established in the above studies, which enable a number of observations to be made regarding the *in vivo* metabolism of corticosterone. The key finding of both pharmacokinetic studies is the rapid clearance of corticosterone, consistent with *in vitro* studies, which demonstrate the hormone, is a high-affinity substrate for the key enzymes required for hepatic clearance (Chapter 4). The magnitude of the difference in rates of metabolism of corticosterone and cortisol by human hepatic cytosol is in keeping with the current *in vivo* data: clearance of corticosterone derived from the bolus study (1.11 l/min) and steady state study (1.46 l/min) proceeds 3-5 times more rapidly than that of cortisol – for which recently reported clearance is consistently between 0.27 and 0.33 L/min (Derendorf et al, 1991; Thomson et al, 2007; Stimson et al, 2009; Perogamvros et al, 2011).

Rapid clearance of corticosterone is reflected in its elimination half-life (t ½), which in both studies is short in comparison to that of cortisol. However, estimates of t ½ differed considerably between bolus and steady state studies, and t ½ was approximately 3-fold longer in the steady state study. Similar differences were seen in V_d. A number of factors might account for these apparent discrepancies. Firstly, assay factors reduce certainty around estimates of circulating tracer concentrations in the bolus study, where concentrations achieved were lower, particularly at later time points. Sample volume was also increased in the steady state study to improve assay

sensitivity. Secondly, administration of tracer as a slow bolus injection results in potential error in modelling the 'peak' of the concentration curve, which was exaggerated due to the shorter than expected t ½. For these reasons, and because of assumptions made in modelling the data to first order kinetics (discussed below), confidence is higher in estimates of pharmacokinetic parameters derived from administration of tracer at steady state rather than as a bolus injection.

Although experimental error due to the above factors might account to some extent for differences in the pharmacokinetic parameters derived from the two studies, biological factors may also be of importance. Unlike clearance, estimated t ½ and V_d for cortisol vary considerably across different studies, with estimated mean t ½ between 60 and 130 minutes in healthy volunteers, and V_d varying from 20 to 50 L (Derendorf et al, 1991; Bright, 1995; Thomson et al, 2007; Perogamvros et al, 2011). Inter-individual variability is also high within the above studies. Reproducible data on these measures is therefore difficult to achieve. Due to this variability, it is difficult to fit glucocorticoid pharmacokinetics to a given compartmental model. In the current studies, a single compartment model has been assumed for the calculation of clearance of a bolus dose; whereas the steady state study has been analysed without compartmental constraints. Previous investigators have variably fitted data to single- or two-compartment models (Toothaker et al, 1982; Thomson et al, 2007; Perogamvros et al, 2011). In the two-compartment model, rapid distribution of free hormone into tissues precedes a slower elimination phase, resulting in non-linear kinetics. Non-linearity also occurs as clearance enzymes approach saturation; although the previously discussed *in vitro* data suggest this is unlikely.

A final factor that requires consideration in interpreting the pharmacokinetic data for D8-corticosterone is the role of plasma protein binding. A relatively high proportion of free: bound hormone is expected to increase V_d and therefore tend to increase t ½ without altering clearance. This phenomenon might explain dose dependent kinetics previously reported for cortisol and corticosterone (Peterson et al, 1960; Toothaker et al, 1982). However, clearance of cortisol has recently been shown to correlate inversely with binding capacity (Perogamvros et al, 2011), via as yet undefined mechanisms. In the current study, bolus pharmacokinetics were assessed near the diurnal peak, when saturation of the high affinity binding protein CBG might be exerting an effect, and therefore capacity for binding of the corticosterone tracer is reduced. Conversely, in the steady-state study the decline in tracer concentration was measured in the early afternoon when occupancy of CBG by endogenous hormone is expected to be lower. Protein binding might therefore influence kinetics according to prevailing concentrations of endogenous glucocorticoid.

The current studies also aimed to estimate the rate of production of corticosterone using the principle of isotope dilution. Although this was achieved, the physiological relevance of the estimate is limited because the high tracer: tracee ratio (required for accurate quantitation of tracer) means that the tracer may have impacted upon the rate of appearance of the tracee. Perhaps related to this, the rate of production is low compared to previous estimates derived from infusion of radiolabelled tracer (Peterson et al, 1960; Huther et al, 1970), although non-specific assay methodology may have over-estimated production rates in these studies.

In addition, urinary corticosterone metabolites were assessed in healthy men. Analysis of urinary steroid profiles has enabled the quantitation of a number of corticosterone metabolites which have not, to our knowledge, previously been quantified. In general, urinary profiles suggest the metabolism of corticosterone mirrors that of cortisol. Tetrahydro-reduced metabolites of both 11-hydroxy- and 11-ketosteroids are by far the most abundant metabolites; and the total excretion rate is broadly proportional to basal plasma concentrations (Zumoff et al, 1974). Our data are broadly consistent with this, with estimated production rates of 6.84 mg/24 hours for cortisol and 0.62 mg/24 hours for corticosterone, corresponding to a corticosterone/cortisol ratio of 8.8 %.

Use of urinary glucocorticoid profiles to determine glucocorticoid production rate is limited by the inability to quantify all urinary metabolites. In the above study this was a particular problem for some 17-deoxymetablites due to the unavailability of standard; and for α-cortol where co-elution prevented quantitation. An additional limitation of using urinary glucocorticoid profiles to determine production rates is the uncertainty regarding completeness of sample collection. Thus the data are mainly useful to provide a qualitative description of corticosterone metabolism, and as an indication of expected ranges in a normal population.

In summary, data presented in the current chapter and chapter 4 demonstrate corticosterone is a high affinity substrate for human hepatic 5β-reductase/3α-HSD and is cleared rapidly *in vivo*. Accurate quantitation of production was not possible due to the high tracer: tracee ratio required for stable isotope tracer studies. Rapid clearance, however, suggests turnover of the hormone is high despite its low

circulating concentration. Altered clearance, as expected during the stress response (Boonen et al, 2013), might therefore affect circulating corticosterone concentrations to a proportionately greater extent than cortisol, Moreover, our data, (section 3.4.3) and that of others (Peterson et al, 1960; Nabors, Jr. et al, 1974; Nishida et al, 1977; Ganguly et al, 1977) show that compared to cortisol, circulating corticosterone concentrations show exaggerated sensitivity to ACTH. In tissues which respond to corticosterone signalling, high turnover of the circulating corticosterone pool may therefore confer sensitivity to rapid changes in HPA axis tone and/or clearance, and the ability to 'fine-tune' corticosteroid receptor activation.

Chapter 7: Corticosterone vs cortisol *in vivo*

7.1. Introduction

The transmembrane protein ABCB1 preferentially exports cortisol over corticosterone, whereas an alternative transporter, ABCC1, exports corticosterone over cortisol (Karssen et al, 2001). The expression of these transporters in humans is known to be tissue specific, with ABCB1 present at the blood-brain barrier (BBB) (Cordon-Cardo et al, 1989), and ABCC1 present in adipose tissue (Figure 1-9). Consistent with selective expression of ABCB1 at the BBB, relative accumulation of corticosterone over cortisol has been demonstrated in the human CNS (Karssen et al, 2001; Raubenheimer et al, 2006). Tissue specific ABC transporter expression therefore provides a mechanism whereby important differences might exist in tissue sensitivity towards corticosterone vs cortisol in the human CNS and adipose tissue.

In the clinical study presented in this chapter, we aimed to test the hypothesis that negative feedback suppression of ACTH is disproportionately sensitive to corticosterone, while adipose tissue is disproportionately sensitive to cortisol. We therefore compared cortisol and corticosterone effects on the HPA axis, adipose tissue and circulating metabolic biomarkers *in vivo* in a random-order crossover study, using ramped steady state infusion to enable assessment of dose-response effect. To minimise confounding by endogenous glucocorticoids, we infused glucocorticoids labelled with stable isotopes – D8-corticosterone (validated in Chapter 4), and the previously validated tracer 9,11,12,12-$[^2H]_4$ cortisol (D4-cortisol) (Andrew et al, 2002) - in individuals with Addison's disease.

7.2. Methods

7.2.1. Ethical and Research Governance Approvals

Approvals were obtained from the South East Scotland Research Ethics Committee (13/SS/0210) and NHS Lothian Research and Development (2013/0305). Research support approvals were obtained from the Wellcome Trust Clinical Research Facility (WTCRF), clinical laboratories, and radiopharmacy at the Royal Infirmary, Edinburgh (RIE).

7.2.2. Participants

Participants with Addison's disease were identified by review of medical records for patients attending endocrinology clinics at the RIE and Western General Hospital (WGH), Edinburgh. Patients were invited to attend a screening visit at the WTCRF, where informed consent was obtained and eligibility to proceed was assessed by means of a medical questionnaire, physical examination and routine blood tests. Weight, height and blood pressure were recorded as described in section 6.2.3.

7.2.2.1. Inclusion criteria

- Addison's disease
- Age > 18 years
- Able to give informed consent
- Alcohol intake < 28 units per week
- Normal FBC, UE, TFT and LFT

7.2.2.2. Exclusion criteria

- Intercurrent illness
- Pituitary disease
- Pregnancy or breastfeeding
- Cardiac, renal or liver failure
- Glucocorticoid treatment within the previous 3 months by any route (other than replacement therapy)
- Uncontrolled hypertension (systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 100 mmHg)
- Blood donation within the preceding 3 months
- Research study participation within the preceding 6 weeks

7.2.3. Sample size

The primary study outcome was plasma ACTH, which was sampled at 10 minute time intervals, and used to calculate mean concentrations over 4 time periods, each lasting 60 - 90 minutes. Frequent time points were chosen to maximise statistical power by reducing variability in mean ACTH arising from ultradian pulsatility. To our knowledge, no similar studies have been undertaken in patients with Addison's disease, but data from healthy volunteers in which ACTH was measured in duplicate at 10 minute intervals following administration of metyrapone (Veldhuis et al, 2001) were used to produce estimates of the mean (49 pmol/l) and standard deviation (6.8 pmol/l) for the primary outcome. The following equation (Van Belle, 2008), derived from Student's unpaired *t*-test was used to calculate sample size (n):

$$n = \frac{\left(z_{1-\alpha/2}+z_{1-\beta}\right)^2(\sigma_1{}^2+\sigma_2{}^2)}{(\mu_1-\mu_2)}$$

Entering the above values for standard deviation (σ) and mean (μ), a sample size of 10 was required for 90 % statistical power (1 – β) at p 0.05 (α), to detect a 20 % difference in the primary outcome.

7.2.4. Clinical protocol

7.2.4.1. Study visits

Participants withheld hydrocortisone from 1400h on the day before each study visit, and omitted fludrocortisone (when prescribed) the morning before the study visit and on the morning of the study visit. They attended the WTCRF, RIE at 0800h following an overnight fast (from 2200h). At t = -15 minutes, intravenous cannulae (18G) were inserted in each antecubital fossa using an aseptic technique. At t = 0, saline infusion (0.9 %, 125 ml/hr) was commenced through the cannula in the left arm, and blood samples collected according to the sampling protocol through the cannula in the right arm. At t = 60, infusion of deuterium-labelled glucocorticoid (D8-corticosterone or D4-cortisol) was commenced according to the steroid infusion protocol (section 7.2.4.3). Order of steroid infusion was chosen randomly, and study participants were blinded to the order of infusate.

At t = 330, biopsy of subcutaneous abdominal adipose tissue was undertaken. Cannulae were then removed, and participants provided with lunch before discharge. After an interval of at least 1 week, participants re-attended for a repeat study visit using the second labelled glucocorticoid.

7.2.4.2. Preparation of the stable isotope tracers D8-corticosterone and D4-cortisol

Deuterium labelled steroids were dissolved in pharmaceutical grade ethanol/water (90/10 %) and filtered in the radiopharmacy to form a sterile stock solution, stored (-40 °C) for a maximum of 8 weeks. Solutions were defrosted and infusions prepared in the clinical research facility on the morning of each study.

7.2.4.3. Steroid infusion protocol

D8-corticosterone (4.18 mg/ml) and D4-cortisol (2.5 mg/ml) stock solutions were prepared in ethanol/water as above (5.5 ml aliquots). Stock solution (5 ml) was added to sodium chloride 0.9% *w/v* (495 ml). At t = 60 mins, a priming dose was administered over 4 minutes, followed by steady state infusion (target concentration 25 nM) for 86 minutes. Further priming doses were administered at t = 140 and 250 mins, each followed by constant infusions, aiming for target concentrations 100 and 250 nM respectively (Table 7-1). For D8-corticosterone, pharmacokinetic parameters derived in the clinical studies described in section 6.4.1.2 were used to calculate priming doses and steady state infusion rates, using calculations described in section 6.2.8.2. The same calculations were used to calculate priming doses and steady state infusion rates for D4-cortisol, using pharmacokinetic data derived from steady state infusion in healthy volunteers (Stimson et al, 2009).

Table 7-1. Corticosterone vs cortisol *in vivo*: priming doses and infusion rates

	D4-cortisol		D8-corticosterone	
Target concentration (nM)	Priming dose (μmol)	Steady state infusion rate (nmol/min)	Priming dose (μmol)	Steady state infusion rate (nmol/min)
25	0.23	3.7	0.65	27.6
100	0.65	17.2	1.95	111.2
250	1.55	51.5	3.89	277.8

7.2.4.4. Sample collection and processing

Samples were obtained in potassium EDTA tubes (2.7 ml) pre-chilled on wet ice and serum gel tubes (9 ml; both Monovette®, Sarstedt, Nümbrecht, Germany). Samples were gently mixed by inverting several times. Potassium EDTA samples were placed on wet ice prior to centrifugation within 30 minutes of sampling; serum gel samples were left at room temperature for 30-45 minutes before centrifugation (1912 × *g* 10 min, 4 °C). Serum/plasma was separated and stored (-80 ºC) for analysis. In plasma, ACTH was quantified by ELISA within 6 weeks of sampling, and NEFAs and glycerol quantified by colorimetric assay (section 2.3). Serum samples were used for quantification of glucocorticoids by LC-MS/MS (Chapter 5); and insulin and glucose by ELISA and colorimetric assay, respectively (section 2.3).

7.2.4.5. Sampling protocol

Potassium EDTA samples were drawn at 10 minute intervals from $t = 0$ (start of saline infusion) until $t = 330$ mins. Serum gel samples were drawn at $t = 0$ & 60 mins; and thereafter at 20 minute intervals until $t = 320$ mins, with additional samples following priming at $t = 70$ & 250 mins (Figure 7-1).

Table 6-3. Daily excretion rate of urinary corticosterone (B) metabolites (17-deoxysteroids) and cortisol (F) metabolites (17 hydroxysteroids) in male volunteers

A = 11-dehydrocorticosterone; THA = 11-dehydro-tetrahydrocorticosterone; THB = tetrahydrocorticosterone; E = cortisone; THE = 5β-tetrahydrocortisone; THF = 5β-tetrahydrocortisol; 5α-isomers designated 'allo'. Standards were not available for 17-deoxy-α-cortolone or 5α--THA; corresponding 17-hydroxylated steroids are not included in data for comparison of total 17 deoxy- and hydroxysteroid excretion.

		Median	Q1	Q3	Min	Max
17-deoxysteroids (µg/24 hours)	11-Dehydrocorticosterone	9.4	7.3	11.2	2.9	19.1
	Corticosterone	11.1	8.1	12.5	2.5	21.5
	THA	106.6	77.9	142.5	38.3	258.6
	THB	70.5	56.8	119.8	39.4	187.6
	Allo-THB	305.9	232.0	462.5	131.9	791.6
	17-deoxy-β-cortolone	31.1	7.1	53.5	0.5	157.3
	17-deoxy-β-cortol	60.0	40.8	75.9	17.8	167.6
17-hydroxysteroids (µg/24 hours)	Cortisone	107.3	86.9	156.8	53.0	183.1
	Cortisol	96.1	83.4	119.1	39.4	161.0
	THE	2989.1	2187.2	3305.5	1794.7	4297.9
	THF	1479.1	1223.2	1733.6	816.3	2033.7
	Allo - THF	1599.5	1024.3	2210.1	493.0	2995.1
	β-Cortolone	394.1	306.9	515.9	253.5	780.9
	α-Cortolone	357.9	278.0	435.9	229.5	868.7
	β-Cortol	354.2	301.7	474.8	182.0	704.5
Total (µg/24 hours)	17-deoxysteroids	615.4	476.5	744.2	291.2	1238.6
	17-hydroxysteroids	6844.1	5753.8	8600.5	4524.7	9627.5
	Ratio 17 deoxy/hydroxy	8.8%	7.7%	11.0%	5.4%	13.3%

6.5. Discussion

The half-life, clearance and volume of distribution of the stable isotope tracer D8-corticosterone following bolus injection and steady-state infusion have been established in the above studies, which enable a number of observations to be made regarding the *in vivo* metabolism of corticosterone. The key finding of both pharmacokinetic studies is the rapid clearance of corticosterone, consistent with *in vitro* studies, which demonstrate the hormone, is a high-affinity substrate for the key enzymes required for hepatic clearance (Chapter 4). The magnitude of the difference in rates of metabolism of corticosterone and cortisol by human hepatic cytosol is in keeping with the current *in vivo* data: clearance of corticosterone derived from the bolus study (1.11 l/min) and steady state study (1.46 l/min) proceeds 3-5 times more rapidly than that of cortisol – for which recently reported clearance is consistently between 0.27 and 0.33 L/min (Derendorf et al, 1991; Thomson et al, 2007; Stimson et al, 2009; Perogamvros et al, 2011).

Rapid clearance of corticosterone is reflected in its elimination half-life (t ½), which in both studies is short in comparison to that of cortisol. However, estimates of t ½ differed considerably between bolus and steady state studies, and t ½ was approximately 3-fold longer in the steady state study. Similar differences were seen in V_d. A number of factors might account for these apparent discrepancies. Firstly, assay factors reduce certainty around estimates of circulating tracer concentrations in the bolus study, where concentrations achieved were lower, particularly at later time points. Sample volume was also increased in the steady state study to improve assay

sensitivity. Secondly, administration of tracer as a slow bolus injection results in potential error in modelling the 'peak' of the concentration curve, which was exaggerated due to the shorter than expected t ½. For these reasons, and because of assumptions made in modelling the data to first order kinetics (discussed below), confidence is higher in estimates of pharmacokinetic parameters derived from administration of tracer at steady state rather than as a bolus injection.

Although experimental error due to the above factors might account to some extent for differences in the pharmacokinetic parameters derived from the two studies, biological factors may also be of importance. Unlike clearance, estimated t ½ and V_d for cortisol vary considerably across different studies, with estimated mean t ½ between 60 and 130 minutes in healthy volunteers, and V_d varying from 20 to 50 L (Derendorf et al, 1991; Bright, 1995; Thomson et al, 2007; Perogamvros et al, 2011). Inter-individual variability is also high within the above studies. Reproducible data on these measures is therefore difficult to achieve. Due to this variability, it is difficult to fit glucocorticoid pharmacokinetics to a given compartmental model. In the current studies, a single compartment model has been assumed for the calculation of clearance of a bolus dose; whereas the steady state study has been analysed without compartmental constraints. Previous investigators have variably fitted data to single- or two-compartment models (Toothaker et al, 1982; Thomson et al, 2007; Perogamvros et al, 2011). In the two-compartment model, rapid distribution of free hormone into tissues precedes a slower elimination phase, resulting in non-linear kinetics. Non-linearity also occurs as clearance enzymes approach saturation; although the previously discussed *in vitro* data suggest this is unlikely.

A final factor that requires consideration in interpreting the pharmacokinetic data for D8-corticosterone is the role of plasma protein binding. A relatively high proportion of free: bound hormone is expected to increase V_d and therefore tend to increase t ½ without altering clearance. This phenomenon might explain dose dependent kinetics previously reported for cortisol and corticosterone (Peterson et al, 1960; Toothaker et al, 1982). However, clearance of cortisol has recently been shown to correlate inversely with binding capacity (Perogamvros et al, 2011), via as yet undefined mechanisms. In the current study, bolus pharmacokinetics were assessed near the diurnal peak, when saturation of the high affinity binding protein CBG might be exerting an effect, and therefore capacity for binding of the corticosterone tracer is reduced. Conversely, in the steady-state study the decline in tracer concentration was measured in the early afternoon when occupancy of CBG by endogenous hormone is expected to be lower. Protein binding might therefore influence kinetics according to prevailing concentrations of endogenous glucocorticoid.

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Use of urinary glucocorticoid profiles to determine glucocorticoid production rate is limited by the inability to quantify all urinary metabolites. In the above study this was a particular problem for some 17-deoxymetablites due to the unavailability of standard; and for α-cortol where co-elution prevented quantitation. An additional limitation of using urinary glucocorticoid profiles to determine production rates is the uncertainty regarding completeness of sample collection. Thus the data are mainly useful to provide a qualitative description of corticosterone metabolism, and as an indication of expected ranges in a normal population.

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Chapter 7: Corticosterone vs cortisol *in vivo*

7.1. Introduction

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7.2.2.1. Inclusion criteria

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- Age > 18 years
- Able to give informed consent
- Alcohol intake < 28 units per week
- Normal FBC, UE, TFT and LFT

7.2.2.2. Exclusion criteria

- Intercurrent illness
- Pituitary disease
- Pregnancy or breastfeeding
- Cardiac, renal or liver failure
- Glucocorticoid treatment within the previous 3 months by any route (other than replacement therapy)
- Uncontrolled hypertension (systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 100 mmHg)
- Blood donation within the preceding 3 months
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7.2.3. Sample size

The primary study outcome was plasma ACTH, which was sampled at 10 minute time intervals, and used to calculate mean concentrations over 4 time periods, each lasting 60 - 90 minutes. Frequent time points were chosen to maximise statistical power by reducing variability in mean ACTH arising from ultradian pulsatility. To our knowledge, no similar studies have been undertaken in patients with Addison's disease, but data from healthy volunteers in which ACTH was measured in duplicate at 10 minute intervals following administration of metyrapone (Veldhuis et al, 2001) were used to produce estimates of the mean (49 pmol/l) and standard deviation (6.8 pmol/l) for the primary outcome. The following equation (Van Belle, 2008), derived from Student's unpaired *t*-test was used to calculate sample size (n):

$$n = \frac{\left(z_{1-\alpha/2}+z_{1-\beta}\right)^2(\sigma_1{}^2+\sigma_2{}^2)}{(\mu_1-\mu_2)}$$

Entering the above values for standard deviation (σ) and mean (μ), a sample size of 10 was required for 90 % statistical power (1 – β) at p 0.05 (α), to detect a 20 % difference in the primary outcome.

7.2.4. Clinical protocol

7.2.4.1. Study visits

Participants withheld hydrocortisone from 1400h on the day before each study visit, and omitted fludrocortisone (when prescribed) the morning before the study visit and on the morning of the study visit. They attended the WTCRF, RIE at 0800h following an overnight fast (from 2200h). At t = -15 minutes, intravenous cannulae (18G) were inserted in each antecubital fossa using an aseptic technique. At t = 0, saline infusion (0.9 %, 125 ml/hr) was commenced through the cannula in the left arm, and blood samples collected according to the sampling protocol through the cannula in the right arm. At t = 60, infusion of deuterium-labelled glucocorticoid (D8-corticosterone or D4-cortisol) was commenced according to the steroid infusion protocol (section 7.2.4.3). Order of steroid infusion was chosen randomly, and study participants were blinded to the order of infusate.

At t = 330, biopsy of subcutaneous abdominal adipose tissue was undertaken. Cannulae were then removed, and participants provided with lunch before discharge. After an interval of at least 1 week, participants re-attended for a repeat study visit using the second labelled glucocorticoid.

7.2.4.2. Preparation of the stable isotope tracers D8-corticosterone and D4-cortisol

Deuterium labelled steroids were dissolved in pharmaceutical grade ethanol/water (90/10 %) and filtered in the radiopharmacy to form a sterile stock solution, stored (-40 °C) for a maximum of 8 weeks. Solutions were defrosted and infusions prepared in the clinical research facility on the morning of each study.

7.2.4.3. Steroid infusion protocol

D8-corticosterone (4.18 mg/ml) and D4-cortisol (2.5 mg/ml) stock solutions were prepared in ethanol/water as above (5.5 ml aliquots). Stock solution (5 ml) was added to sodium chloride 0.9% *w/v* (495 ml). At t = 60 mins, a priming dose was administered over 4 minutes, followed by steady state infusion (target concentration 25 nM) for 86 minutes. Further priming doses were administered at t = 140 and 250 mins, each followed by constant infusions, aiming for target concentrations 100 and 250 nM respectively (Table 7-1). For D8-corticosterone, pharmacokinetic parameters derived in the clinical studies described in section 6.4.1.2 were used to calculate priming doses and steady state infusion rates, using calculations described in section 6.2.8.2. The same calculations were used to calculate priming doses and steady state infusion rates for D4-cortisol, using pharmacokinetic data derived from steady state infusion in healthy volunteers (Stimson et al, 2009).

Table 7-1. Corticosterone vs cortisol *in vivo*: priming doses and infusion rates

	D4-cortisol		D8-corticosterone	
Target concentration (nM)	Priming dose (μmol)	Steady state infusion rate (nmol/min)	Priming dose (μmol)	Steady state infusion rate (nmol/min)
25	0.23	3.7	0.65	27.6
100	0.65	17.2	1.95	111.2
250	1.55	51.5	3.89	277.8

7.2.4.4. Sample collection and processing

Samples were obtained in potassium EDTA tubes (2.7 ml) pre-chilled on wet ice and serum gel tubes (9 ml; both Monovette®, Sarstedt, Nümbrecht, Germany). Samples were gently mixed by inverting several times. Potassium EDTA samples were placed on wet ice prior to centrifugation within 30 minutes of sampling; serum gel samples were left at room temperature for 30-45 minutes before centrifugation (1912 × *g* 10 min, 4 °C). Serum/plasma was separated and stored (-80 ºC) for analysis. In plasma, ACTH was quantified by ELISA within 6 weeks of sampling, and NEFAs and glycerol quantified by colorimetric assay (section 2.3). Serum samples were used for quantification of glucocorticoids by LC-MS/MS (Chapter 5); and insulin and glucose by ELISA and colorimetric assay, respectively (section 2.3).

7.2.4.5. Sampling protocol

Potassium EDTA samples were drawn at 10 minute intervals from $t = 0$ (start of saline infusion) until $t = 330$ mins. Serum gel samples were drawn at $t = 0$ & 60 mins; and thereafter at 20 minute intervals until $t = 320$ mins, with additional samples following priming at $t = 70$ & 250 mins (Figure 7-1).

7.2.4.6. Biopsy of subcutaneous abdominal fat

Biopsy of subcutaneous abdominal fat was undertaken using an aseptic technique from a site approximately 10 cm lateral to the umbilicus. Following injection of local anaesthetic (2 % lignocaine, 5 ml, Hameln Pharmaceuticals, Gloucester, UK), a 14-gauge needle attached to a 60 ml syringe was inserted subcutaneously, directed towards the umbilicus. The plunger was withdrawn to create a vacuum, and held in place using a 13 x 100 mm glass culture tube while a sample of adipose tissue was obtained (using two passes). Samples were placed on autoclaved Isopon^{TM} aluminium mesh (DF Wishart, Edinburgh, UK), washed with DEPC water, then placed on dry ice before storage (-80 °C) for analysis of glucocorticoid-responsive gene expression by qRT-PCR (section 2.3.8). Samples were not obtained from subjects in whom the procedure was deemed to be unsafe (due to low adiposity).

7.3. Data Analysis

LC-MS/MS data were analysed as described in section 5.5.4. For the quantification of D4-cortisol, which is converted to D3-cortisol *in vivo* (Andrew et al, 2002), interference from [M+4] isotopomers of cortisol was corrected for by adjusting D4-cortisol peak area according to the abundance of [M+4] cortisol in a standard of D3-cortisol (approximately 13 %; calculated for each assay). A similar correction was applied to adjust D3-cortisol for the abundance of [M+3] cortisol isotopomers in a standard of D4-cortisol (approximately 4 %). Naturally occurring [M+3] and [M+4] isotopomers of unlabelled cortisol were detected at less than 2 %, and were not adjusted for.

In the analysis of glucocorticoid-responsive biomarkers (ACTH, glucose, insulin, NEFAs and glycerol), to account for differences in steady state concentrations of labelled cortisol vs corticosterone, analyses were performed both unadjusted and adjusted for steady-state concentration of infused steroid. Mean changes in circulating concentration are expressed as % change from baseline (t = 0-60 mins). In analyses adjusted for steroid concentration, each steroid was coded as a categorical variable (cortisol = 0; corticosterone = 1) and entered into a multiple linear regression model as a co-variable with steady state concentration of labelled steroid. Response variable was % change in a given circulating glucocorticoid-responsive biomarker. The validity of the linear regression model was confirmed by analysis of histograms and normal plots of residuals.

Relative gene abundance (RGA) in subcutaneous adipose biopsy was adjusted for differences in steady-state steroid concentration over the duration of each study visit by calculating area under the curve (AUC) for infused D4-cortisol (D4-F) and D8-corticosterone (D8-B) to produce a correction factor for each participant, used in the following equation:

$$Adjusted\ RGA\ (D4\text{-}F) = RGA\ (D4\text{-}F) \times \frac{AUC\ (D8\text{-}B)}{AUC\ (D4\text{-}F)}$$

Student's paired t-test was used for all comparisons of means between study phases. All statistical analyses were performed using Minitab (Version 17; State College PA).

7.4. Results

7.4.1. Characteristics of study participants

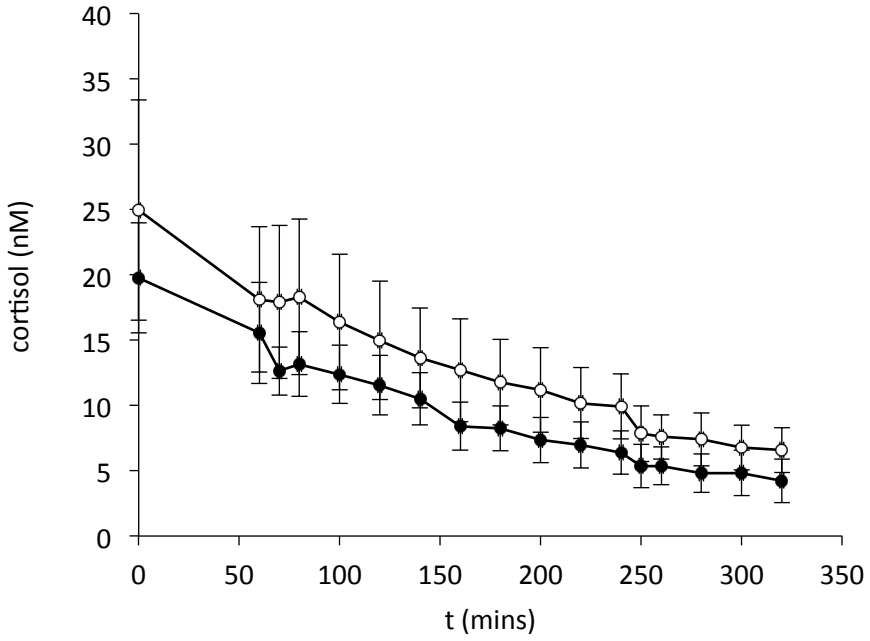
Of 10 study participants, one was excluded from all analyses because baseline ACTH in both study visits (2.8 & 4.0 pmol/l) and baseline endogenous cortisol concentration on one study visit (533 nM) suggested the diagnosis of primary adrenal insufficiency was incorrect, and/or hydrocortisone replacement had not been withheld as per study protocol. Characteristics of the remaining study participants (n = 9) are summarised in Table 7-2, and concentrations of endogenous cortisol are presented in Figure 7-2. Concentrations of endogenous corticosterone were too low to enable quantification.

Table 7-2. Corticosterone v cortisol *in vivo*: characteristics of study participants (n = 9; 3 male)

	Mean ± standard deviation	Range
Age (years)	53 ± 14	(20 - 65)
BMI (kg/m^2)	24.7 ± 3.5	(21.7 - 32.9)
Years since diagnosis	19 ± 10	(4 - 30)
Daily hydrocortisone dose (mg)	21 ± 6	(15 - 30)
Daily fludrocortisone dose (µg)	76 ± 91	(0 - 300)

Figure 7-2. Endogenous cortisol in patients with Addison's disease during infusion of deuterated glucocorticoids

Serum endogenous cortisol concentration during infusion of D8-corticosterone (open circles) or D4- cortisol (closed circles) in patients with Addison's disease (n = 9; total study visits = 18).

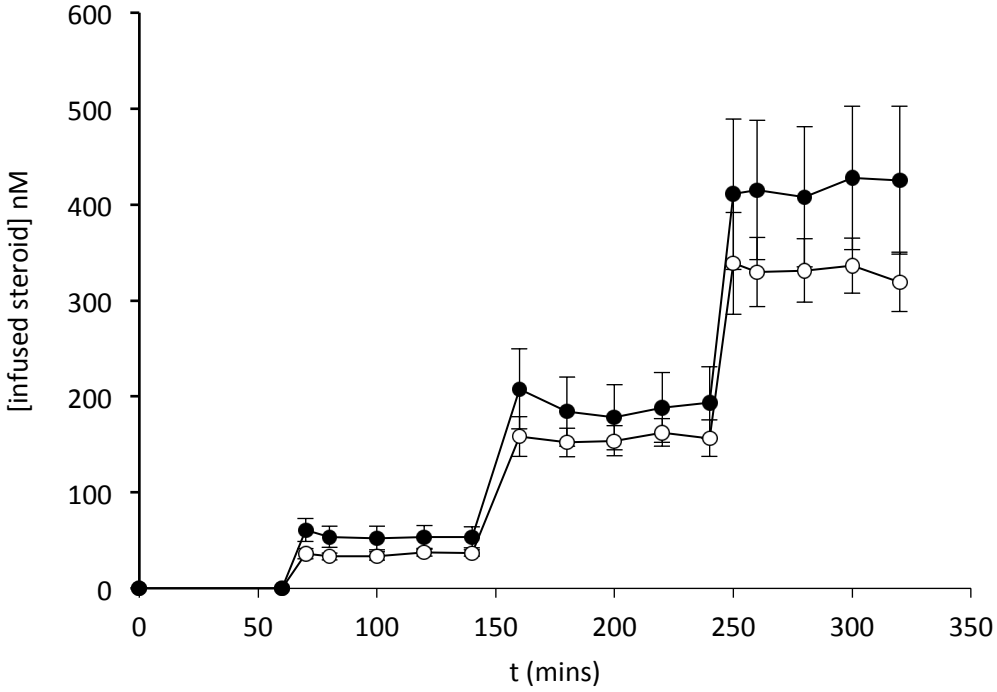


7.4.2. Serum concentration of deuterium-labelled glucocorticoids

Assessment of circulating concentrations of labelled cortisol (sum of D3-cortisol + D4-cortisol) and corticosterone indicated ramped steady state had been achieved with appropriate priming (Figure 7-3). Steady state concentrations achieved tended to be higher for cortisol than corticosterone, although the differences were not statistically significant.

Figure 7-3. Serum concentration of deuterium labelled corticosterone and cortisol

Total deuterated cortisol (closed circles) = sum [D3-cortisol] + [D4-cortisol]. Open circles = D8-corticosterone. Data in table refer to mean ± SEM concentration (5 measurements at each steady-state). P value refers to Student's paired t –test.



t (mins)	[D4–cortisol] + [D3-cortisol] nM	[D8-corticosterone] nM	p
80-140	53.2 ± 11.5	35.4 ± 4.0	0.14
160-240	190.5 ± 37.0	156.6 ± 14.7	0.37
260-330	423.6 ± 74.4	331.1 ± 32.0	0.22

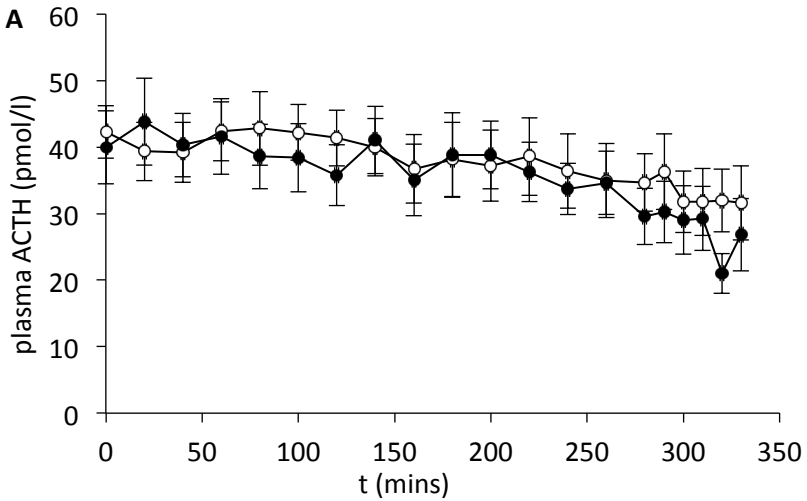
7.4.3. Plasma ACTH concentration

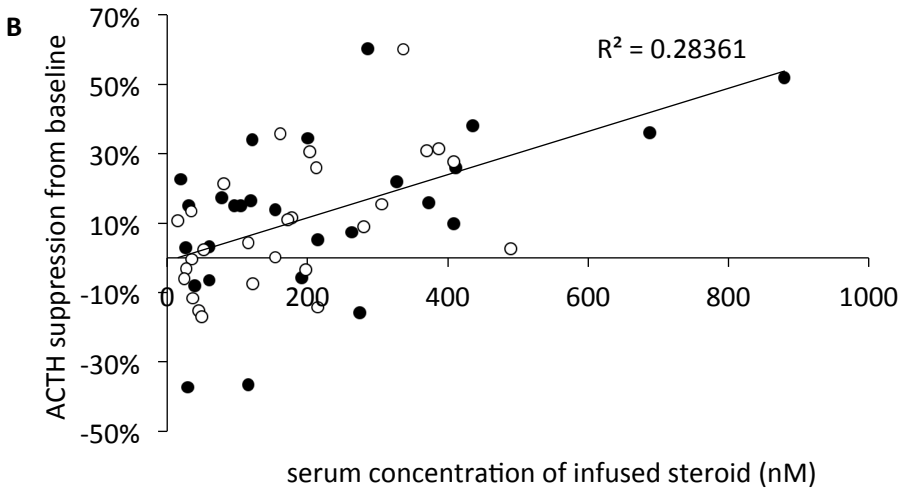
Change in plasma ACTH for all subjects according to infused steroid is shown in Figure 7-4. ACTH at baseline was elevated but did not differ between study phases (41.4 ± 2.7 vs 40.9 ± 2.1 pmol/l in cortisol vs corticosterone groups respectively; p = 0.6; assay reference range 0.2 – 15.8 pmol/l). In multiple linear regression analysis,

serum concentration of infused steroid was positively associated with degree of suppression of plasma ACTH from baseline ($\beta = 0.062 \pm 0.014$; $p < 0.0001$). The coded categorical variable (D4-cortisol = 0; D8-corticosterone = 1) was not a significant predictor variable ($\beta = -0.22 \pm 0.49$; $p = 0.96$), indicating ACTH suppression did not differ significantly between D4-cortisol and D8-corticosterone infusion.

Figure 7-4. Plasma ACTH concentration during infusion of D4-cortisol and D8-corticosterone

Plasma ACTH during infusion of saline (t = 0-60) followed by ramped steady state infusion of D4-cortisol (D4-F; closed circles) and D8-corticosterone (D8-B; open circles). Each data point in **B** represents mean % suppression of ACTH during each of three steady states (t = 80-140, 160-240 and 260-330) for each participant. Table i: ACTH suppression during D4-F and D8-B infusion in analysis unadjusted for steroid concentration. Table ii: Each steroid coded as a binary variable and entered into a multiple linear regression equation in analysis adjusted for steroid concentration. P values refer paired t-test (table i) and partial regression co-efficient (β; table ii).





(i) Unadjusted	% Suppression of ACTH from baseline		
t (mins)	D4-F	D8-B	p
80-140	2.6 % ± 6 %	-3 % ± 4 %	0.43
160-240	8.3 % ± 7 %	10 % ± 5 %	0.86
260-330	28.2 % ± 8 %	23 % ± 6 %	0.43

(ii) Adjusted				
Co-variables				R^2
Concentration of infused steroid		D4-F (1) vs D8-B (0)		
β	p	β	p	
0.062 ± 0.014	< 0.001	-0.22 ± 4.89	0.96	28.4 %

7.4.4. Metabolic markers of glucocorticoid action

Circulating concentrations of glucose, insulin, glycerol and NEFAs are shown in Figure 7-5. Rise in plasma NEFA concentration was positively associated with concentration of infused steroid ($\beta = 0.11 \pm 0.05$, $p = 0.01$) but did not differ significantly during D4-cortisol vs D8-corticosterone infusion in analyses unadjusted (51.3 ± 26.3 % vs 26.5 ± 15.2 %, $p = 0.17$) or adjusted ($\beta = -19.0 \pm 16.7$, $p = 0.26$) for steroid concentration. Changes in concentration of all other analytes (glucose, insulin or glycerol) were not significantly associated with concentration of steroid infused and did not vary according to administration of D4-cortisol or D8-corticosterone (Table 7-3).

Figure 7-4. Circulating metabolic markers during glucocorticoid infusion

Circulating glucose (A), insulin (B), glycerol (C) and non-esterified fatty acids (NEFAs; D) during infusion of saline (t = 0 – 60) then D4-cortisol (closed circles) or D8-corticosterone (open circles).

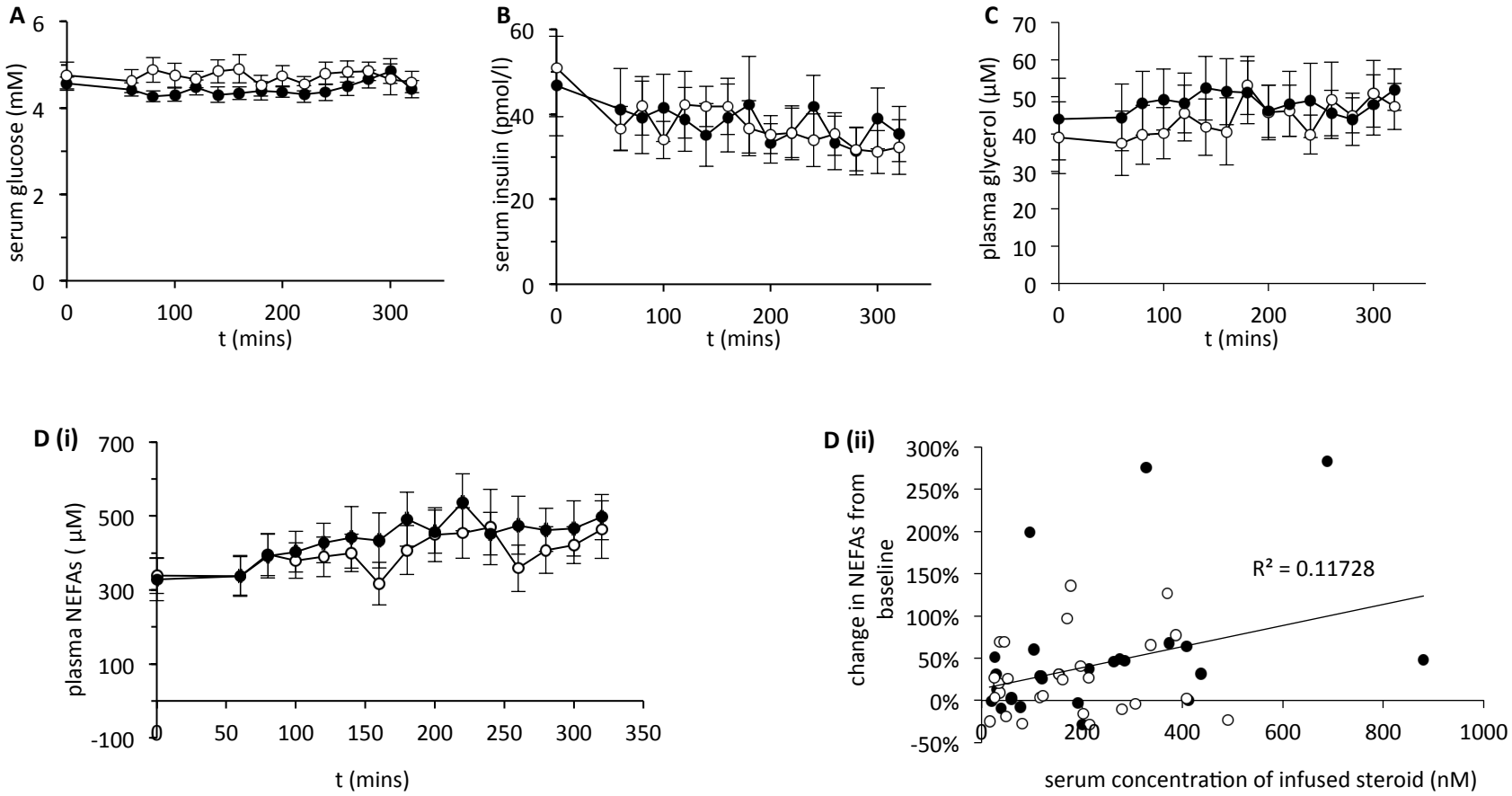


Table 7-3. Changes in circulating metabolic markers during infusion of D4-cortisol or D8-corticosterone

(i) % Change in circulating glucose, insulin, non-esterified fatty acids (NEFAs) and glycerol from baseline (0-60 mins) during D4-cortisol (D4-F) and D8-corticosterone (D8-B) infusion in analysis unadjusted for steroid concentration. (ii) Each steroid coded as a binary variable and entered into a multiple linear regression equation in analysis adjusted for steroid concentration. P values refer paired t-test (table i) and partial regression co-efficient (β; table ii).

(i) Unadjusted analysis

	t (mins)	% Change from baseline		p
		D4-F	D8-B	
Glucose	80-140	-3.2% ± 3.4%	-3% ± 4%	0.14
	160-240	-2.6% ± 2.8%	4.3% ± 3.1%	0.31
	260-330	3.3% ± 4.4%	2.7% ± 3.1%	0.91
Insulin	80-140	-5.4% ± 4.5%	-3.0% ± 7.0%	0.73
	160-240	-5.4% ± 8.0%	-8.3% ± 7.6%	0.74
	260-330	-12.9% ± 8.1%	-16.2% ± 8.0%	0.74
Glycerol	80-140	19.7% ± 10.5%	16.2% ± 7.2%	0.62
	160-240	19.9% ± 12.5%	42.1% ± 34.7%	0.54
	260-330	16.1% ± 11.4%	66.0% ± 63.2%	0.20
NEFAs	80-140	35.2% ± 21.4%	20.1% ± 11.1%	0.36
	160-240	56.9% ± 28.4%	29.7% ± 18.2%	0.15
	260-330	60.4% ± 29.5%	28.8% ± 17.2%	0.15

(ii) Adjusted for infused steroid

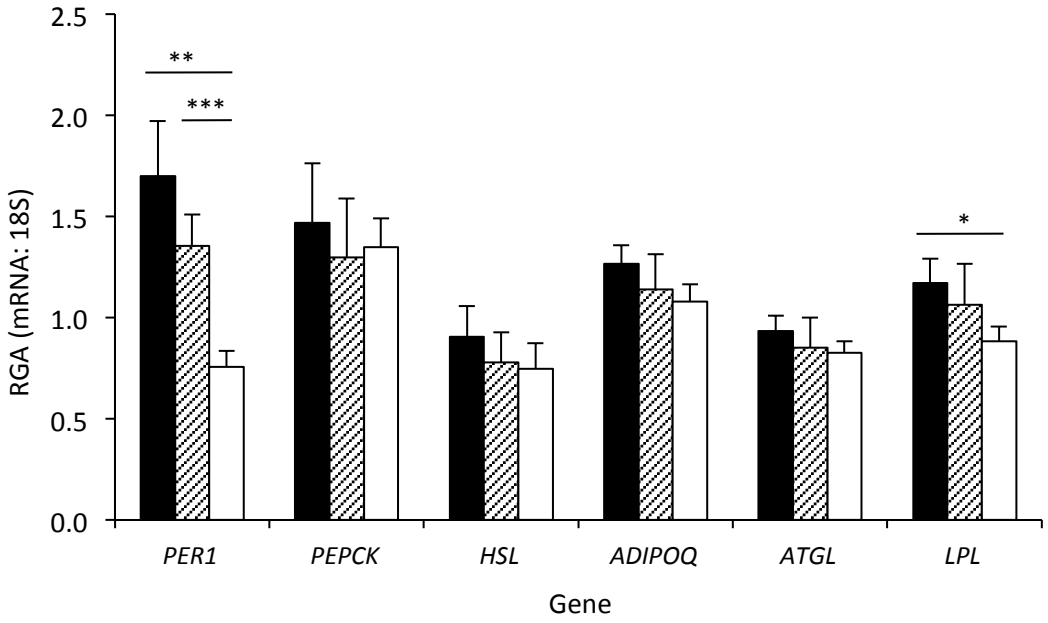
	Co-variables				
	Concentration of infused steroid		D4-F (1) vs D8-B (0)		
Dependent variable	β	p	β	p	R^2
Glucose	-0.002 ± 0.014	0.81	3.29 ± 2.90	0.26	3.0 %
Insulin	-0.023 ± 0.017	0.64	-2.43 ± 5.89	0.68	3.8 %
Glycerol	-0.011 ± 0.021	0.60	-10.2 ± 7.74	0.19	4.1 %
NEFAs	0.117 ± 0.048	0.02	-19.0 ± 16.7	0.26	13.9 %

7.4.5. Glucocorticoid-responsive gene expression in subcutaneous adipose tissue

Relative expression of glucocorticoid responsive genes in biopsies of subcutaneous adipose tissue sampled following infusion of D4-cortisol or D8-corticosterone are compared in Figure 7-6. Abundance of *PER1* mRNA was greater following D4-cortisol than D8-corticosterone (2.2 fold; $p < 0.01$). This association was attenuated, but more statistically robust (1.8 fold; $p < 0.001$), following adjustment for differences in concentration of infused steroid. No statistically significant differences were seen in the relative abundance of other gene transcripts analysed, with the exception of *LPL*, which was more abundant following D4-cortisol infusion (1.3 fold, $p = 0.03$), but not after correction for concentration of infused steroid.

Figure 7-6. Relative expression of glucocorticoid responsive genes in subcutaneous adipose tissue following infusion of D4-cortisol or D8-corticosterone.

Relative gene abundance (RGA; normalised to expression of 18S rRNA) in subcutaneous adipose biopsy tissue, following D4-cortisol (D4-F; closed bars) or D8-corticosterone infusion (D8-B; open bars). D4-F concentration, expressed as area under the curve (AUC) was higher than D8-B (60194 ± 11047 vs 47099 ± 4467 nM.min. RGA following D4-F infusion was therefore adjusted (hatched bars) as follows:
Adjusted RGA (D4-F) = RGA (D4-F) × (AUC (D8-B))/(AUC (D4-F)). P values refer to Student's paired t-test for comparison of RGA following D8-B vs D4-F, adjusted or unadjusted for differences in concentration. *p < 0.05, ** p < 0.01, *** p < 0.001.



Gene	RGA (D4-F infusion)		RGA (D8-B infusion)	p (unadjusted)	p (adjusted)
	Unadjusted	Adjusted			
PER1	1.70 ± 0.27	1.35 ± 0.16	0.76 ± 0.08	<0.01	<0.001
PEPCK	1.47 ± 0.29	1.30 ± 0.29	1.35 ± 0.14	0.61	0.82
HSL	0.91 ± 0.15	0.78 ± 0.15	0.75 ± 0.13	0.24	0.81
ADIPOQ	1.27 ± 0.09	1.14 ± 0.18	1.08 ± 0.09	0.17	0.76
ATGL	0.93 ± 0.08	0.85 ± 0.15	0.83 ± 0.05	0.26	0.87
LPL	1.17 ± 0.12	1.06 ± 0.20	0.88 ± 0.07	0.03	0.45

7.5. Discussion

In a random-order crossover study of patients with Addison's disease, we have demonstrated that gene expression in adipose tissue responds disproportionately sensitivity to cortisol is enhanced relative to corticosterone, using doses which are equipotent for ACTH suppression. Changes in adipose gene expression were not, however, reflected in systemic metabolic markers, and suppression of ACTH by both glucocorticoids was less than expected.

We chose to study patients with Addison's disease to minimise confounding due to endogenous glucocorticoids. Pharmacokinetic studies in healthy volunteers enabled accurate priming and closely matched steady state concentrations of each glucocorticoid. The dosing protocol was designed to achieve glucocorticoid concentrations representative of those occurring at varying times in the diurnal cycle, and to allow a dose-response assessment to account for differences in serum concentrations between groups and individuals.

As expected in Addison's disease, ACTH was elevated before glucocorticoid administration. However, suppression was incomplete, and in the final steady state, where glucocorticoid concentrations were approximately equivalent to those at the cortisol diurnal peak, ACTH remained elevated. This finding is of interest and suggests the HPA axis is to an extent 'resistant' to negative feedback control in Addison's disease. Suppression of ACTH in Addison's disease has been described as normal (Besser et al, 1971), although generally where 'physiological' doses of steroid are used (Holdaway, 1973; Clayton et al, 1977; Feek et al, 1981) incomplete

suppression is reported. The situation may be analogous to that of the hypothalamo-pituitary-thyroid axis, where TSH fails to suppress to the normal range following acute administration of thyroid hormone in treatment-naïve patients with primary hypothyroidism (Clayton et al, 1977). The mechanism behind abnormal feedback control of the HPA axis in primary adrenal insufficiency has not been established. However, periods of glucocorticoid deficiency are likely to occur in treated Addison's disease due to inadequacies in the pharmacokinetics of oral hydrocortisone (Debono et al, 2009; Forss et al, 2012; Grossman et al, 2013), and these may result in a degree of autonomous function and/or hyperplasia of corticotrophs. Consistent with this, a number of reports describe persistently elevated ACTH in Addison's disease accompanied by corticotroph hyperplasia (Miyabo et al, 1990) or adenomata (Jara-Albarran et al, 1979; Krautli et al, 1982; Yanase et al, 1985; Sugiyama et al, 1996). The development of autonomous ACTH secretion in Addison's disease may therefore be analogous to Nelson's syndrome, in which glucocorticoid deficiency contributes to growth of ACTH-producing pituitary tumours following bilateral adrenalectomy (Barber et al, 2010).

Our data do not provide clear evidence to support the hypothesis that the presence of ABCB1 at the blood-brain barrier (BBB) renders the feedback centres of the CNS differentially sensitive to corticosterone over cortisol. It remains possible that ABCB1 enhances sensitivity of the hippocampus and hypothalamus to corticosterone, but not the pituitary, which is outside the BBB. In our study, corticosteroid receptors in the hippocampus and hypothalamus, thought to be predominantly high-affinity MR (de Kloet et al, 1998) may have been saturated

during the final steady state. Limited suppression of ACTH at lower glucocorticoid concentrations may have reduced the power of our study to detect differences in feedback at lower glucocorticoid concentrations, and it remains possible that in individuals without primary adrenal insufficiency differential feedback occurs outwith the diurnal peak. However, where clear changes in ACTH are seen towards the end of our study, the absence of significant differences during infusion of each steroid, despite steady state concentrations of cortisol which tended to be higher than those of corticosterone, suggest the effects of corticosterone on the HPA axis is at least equivalent in potency to those of cortisol.

In contrast, data from subcutaneous adipose tissue demonstrate potentially important differences. Expression of the glucocorticoid-responsive gene *PER1* during cortisol infusion was approximately two-fold greater than during corticosterone infusion, and enhanced expression of *LPL* was also seen following cortisol infusion, although slight differences in serum steroid concentrations make the latter finding difficult to interpret. *PER1* encodes period circadian homolog 1, a key component of the circadian clock in peripheral tissues, disruption of which is associated with metabolic dysregulation (Eckel-Mahan et al, 2013). *PER1* is rapidly induced by glucocorticoids (Stavreva et al, 2009) and uniquely sensitive at low doses which do not alter expression of other 'glucocorticoid-responsive' genes (Reddy et al, 2012). The absence of differences in expression of other genes in our study may reflect an inadequate period of time for glucocorticoids, which turn over slowly in human adipose tissue (Hughes et al, 2010), to equilibrate. The relatively small changes in circulating metabolic markers may also be explained by this phenomenon; previous

investigators have found plasma glucose, insulin and glycerol show a small and delayed response to several hours of 'physiological' glucocorticoid infusion, and although the rise in NEFAs generally occurs more rapidly, there is wide inter- and intra-individual variability (Dinneen et al, 1993; Djurhuus et al, 2002). Our data also needs to be interpreted in the light of the limtations of qRT-PCR, which does not quantify protein product.

A number of factors are potentially involved in differential tissue sensitivity to cortisol and corticosterone. These include glucocorticoid receptor affinity, although this is thought to be similar for both glucocorticoids (Veldhuis et al, 1982; Krozowski et al, 1983; Sutanto et al, 1987; Reul et al, 1990); and pre-receptor metabolism by 11β-HSD1, which may amplify cortisol action to a greater extent than corticosterone (Maser et al, 2002; Arampatzis et al, 2005). Modulation of glucorticoid sensitivity by transmembrane export has received very little research attention. Export of corticosterone by ABCC1, known to be highly expressed in adipose tissue, may explain our findings, with expression of alternative transporters in the BBB resulting in retained sensitivity to corticosterone in the CNS. Regardless of the underlying mechanism, the concept of differential tissue sensitivity to multiple endogenous glucocorticoids is a novel one, with potentially important clinical implications. Corticosterone, which may induce a limited metabolic response relative to that in the CNS, offers promise as a therapeutic tool to optimise CNS glucocorticoid action whilst limiting adverse metabolic effects of glucocorticoid replacement.

Chapter 8: Conclusions

The studies presented in this thesis are the first to explore in detail the role of corticosterone, the 'neglected second glucocorticoid', in human health and disease. In earlier chapters, I have described the development of a robust LC-MS/MS assay for corticosterone, the validation of a novel stable isotope tracer, and experiments that provide key data on the 'pharmacokinetics' of corticosterone in humans. These studies have provided the tools required to design a clinical study that has broken new ground by infusing corticosterone in humans in a manner that enables direct comparison with cortisol.

The data indicate differences in a number of the key physiological properties of corticosterone and cortisol. We have demonstrated rapid clearance of corticosterone in comparison to cortisol *in vivo*, consistent with our *in vitro* studies. Together these show approximately 3-fold differences in clearance, half-life and rate of hepatic metabolism between two endogenous glucocorticoids. It is increasingly recognised that the HPA axis is regulated by changes in glucocorticoid clearance in addition to production. Given the rapid clearance of corticosterone, a relative reduction in the activity of clearance enzymes common to both glucocorticoids may be raise plasma corticosterone to a greater extent than cortisol. We have also shown corticosterone is disproportionately sensitive to ACTH stimulation. During times of rapid changes in HPA axis tone, and/or clearance, the corticosterone pool may be of particular importance. Corticosterone has largely been neglected in humans because of it low basal circulating concentrations, yet it is clear this is only one of a number of parameters which require consideration when studying glucocorticoid physiology.

Key among these parameters, with respect to the findings of this thesis, is the relative affinities of each glucocorticoid for ABC transporter proteins, which have recently been shown to differ for cortisol and corticosterone. We have shown that corticosterone is no less potent than cortisol in suppressing ACTH. In contrast, mRNA expression in adipose tissue is disproportionately sensitivity to cortisol over corticosterone. The data are the first to suggest differential sensitivity to endogenous glucocorticoids in humans. Findings in adipose tissues are consistent with known expression of *ABCC1*; while in the CNS we hypothesised ABCB1 would confer sensitivity to corticosterone over cortisol. The failure to detect this may however reflect inadequate sensitivity of ACTH as marker of acute glucorticoid action in the CNS in Addison's disease.

Confirmation of differential adipose tissue sensitivity towards corticosterone vs cortisol may have important clinical implications. Elevated cortisol action in adipose tissue is implicated in the pathogenesis of metabolic syndrome, a collection of major risk factors for cardiovascular disease. Our data from the East Hertfordshire cohort show that those individuals who have a 'steroidogenic bias' producing relatively high levels of circulating cortisol and low levels of corticosterone under ACTH stimulation have an adverse metabolic profile. It is likely that the activity of CYP17 is a key determinant of this 'bias'. Furthermore, cortisol excess, driven by high CYP17 activity, may be compounded by relative corticosterone deficiency, which drives activation of the HPA axis. In these individuals, corticosterone therapy might allow control of HPA axis activation without inducing adverse metabolic effects.

This concept might also be of importance for individuals with glucocorticoid deficiency. Current glucocorticoid replacement strategies fail to optimise CNS function, whilst risking peripheral glucocorticoid toxicity (Grossman et al, 2013). Even in the absence of confirmed CNS sensitivity to corticosterone over cortisol, enhanced adipose tissue sensitivity to cortisol over corticosterone would imply that, at equivalent doses, metabolic side-effects might be less with corticosterone therapy in comparison to cortisol.

The short half-life of corticosterone means that modification of its formulation, similar to that currently being undertaken for cortisol (Johannsson et al, 2009; Verma et al, 2010), would be required for oral administration. However, interest has arisen recently in subcutaneous glucocorticoid replacement (Oksnes et al, 2014), which could be employed in a proof-of-principle study to compare effects of corticosterone and cortisol on ACTH and metabolic parameters assessed over a longer time-period than that of the current study. The use of functional MRI also offers a promising means of assessing the CNS effects of glucocorticoids at physiological doses in humans (Lovallo et al, 2010; Sudheimer et al, 2013), perhaps providing an additional CNS biomarker for use in future studies.

A number of other questions are raised by our studies, which deserve further attention. These include whether the distinct properties of corticosterone, with rapid clearance and response to ACTH, are of physiological relevance. One area where this may be the case is that of ultradian regulation of the HPA axis, the significance of which is increasingly recognised (Young et al, 2004; Stavreva et al, 2009). Mathematical modelling, using kinetics derived from rodents, suggests ultradian

pulsatility is an intrinsic product of feedback and delayed feed-forward signalling between the pituitary and adrenal glands (Walker et al, 2010). Due to its long half-life, cortisol signalling alone may not explain pulsatility in the human HPA axis; whereas for corticosterone, higher-frequency oscillations are possible, which may be key for pulsatile ACTH release in humans.

Secondly, following on from our epidemiological studies, it would of interest to assess associations of variability in *CYP17A1*, encoding 17α-hydroxylase, with plasma corticosterone and cortisol levels. Polymorphism in *CYP17A1* has been previously been associated with hypertension (Newton-Cheh et al, 2009), obesity (Yan et al, 2012; Hotta et al, 2012), and coronary artery disease (Schunkert et al, 2011), but the mechanisms behind these associations have not been established. Studies to assess the functional relevance of polymorphism in *CYP17A1*, and to determine whether associations with metabolic syndrome are mediated by relative production of corticosterone and cortisol, may be fruitful.

Finally, while we hypothesise that our results in the study of patients with Addison's disease are explained by corticosterone export from adipose tissue by ABCC1, it would be of interest to test this hypothesis by repeating the study using specific inhibitors of transport function. ABC transporter inhibitors previously used in humans have relatively high toxicity (Pennock et al, 1991; Eyal et al, 2010), although more recently discovered agents appear to be better tolerated (Bauer et al, 2012).

Ongoing investigation of these questions will continue to challenge the assumption that a single endogenous glucocorticoid is of importance in humans. Our findings

raise the possibility of distinct roles for corticosterone and cortisol in human health and disease. Corticosterone may optimise glucocorticoid action in the human CNS, and simultaneously limit adverse metabolic effects driven by cortisol excess.

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